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The University of Bergen School of Medicine the Gade Institute Department of Pathology Bergen Norway Head Professor E Waaler M D

STUDIES IN HYPOTHERMIA

4 *The Influence of Hypothermia on the Degranulation of Mesenteric Mast Cells in Turpentine Peritonitis in Mice*

By

KNUT SVANES

Received 11 i 65

The influence of deep hypothermia on acute turpentine inflammation in mice has been studied previously (*Svanes* 1964 a and b) It was shown that the initial oedema formation was reduced and that the emigration of blood leucocytes was strongly retarded under hypothermia

The onset of acute inflammation in the skin peritoneum and pleura of normal rats is associated with degranulation of the tissue mast cells (*Maximow* 1904 *Ernst* 1926 *McGovern* 1957, *Gustafsson & Cronberg* 1957 *Spector & Willoughby* 1959 *Sheldon & Bauer* 1960), and this degranulation of the mast cells is associated with release of substances which increase the capillary permeability

The present paper describes the effect of hypothermia on the degranulation of mast cells in acute turpentine peritonitis in mice

MATERIALS AND METHODS

Animals 104 female albino mice which were 4 6 months old and weighed 24 0 + 2 0 g were used They were distributed at random in groups of 4 mice

Acute peritonitis was produced by intraperitoneal injection of turpentine partly undiluted in single doses of 0 02 ml partly as 25 and 5 per cent solutions in olive oil

p

s were counted in the fat free peritoneal windows of th mesentery In each mouse about 100 mast cells were counted in each of the 3 windows which showed the greatest number of degranulated cells Mast cells occurring in clumps were not counted since it was difficult to judge degranulation

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in these cells The mast cells were devided into different cell types according to the degree of degranulation as described by *Gustafsson & Cronberg* (1957) A-cells = normal mast cells with no ejected granules B-cells = cells with 1 10 ejected granules C-cells = cells surrounded by granules but with the central part intact D-cells = totally disrupted mast cells with the granules evenly scattered (see Fig 1)

Anaesthesia and hypothermia Sodium penth barbital was used for anaesthesia as described in detail previously (Stanes 1964 a)

The body temperature remained normal when the anaesthetized mice were kept in a thermostat at 32-34° C

Hypothermia was induced by placing anaesthetized mice in a *refrigerator* at 4° C. After 6 minutes at 4° C the rectal temperatur had fallen to about 27° C The temperature remained about 27° C when the mice were transferred to a thermostat at [illegible]

[illegible] temperature had been reached

EXPERIMENTS AND RESULTS

The Effect of Barbiturate Anaesthesia on the Degranulation of Mast Cells Caused by Turpentine

Eight groups of mice were used (Table 1) Groups 1–4 were kept under normal conditions during the experiment Groups 5 8 were anaesthetized and kept at normal body temperature The mice were given an intraperitoneal injection of turpentine *Table* 1 shows the concentration of turpentine which was *used for the different* groups For groups 5 8 the injection was given after 1 hour of anaesthesia The mice *were killed 45* minutes after the injection of turpentine

The mast cell counts are recorded in Table 1 *A two way analysis of* variance was performed for each type of mast cells Arcsin transformation was used

The mice treated with undiluted *turpentine* (groups 1 and 5) showed marked degranulation of the *mast cells* The mice treated with 25 per cent turpentine (groups 2 and 6) showed moderate degranulation of the mast cells, apart *from* 1 case in group 2 where [illegible]

[illegible] with 5 per cent turpentine (*groups 3 and* 7) showed normal distribution of the *mast* cells, apart *from* 1 case in *group 3 where* the count disclosed a high degree of degranulation (A cells 1 *per cent*, B cells 2 per cent, C cells 45 per cent, D cells 52 *per cent*) *The* effect of 5 per cent turpentine did not differ significantly *from that* of pure olive-oil for any cell type

The distribution of mast *cells in the* anaesthetized groups was about the same as that in the *corresponding* control groups The statistical analyses disclosed no *significant effect* of anaesthesia, and no significant interaction between *anaesthesia* and concentration of *turpentine*

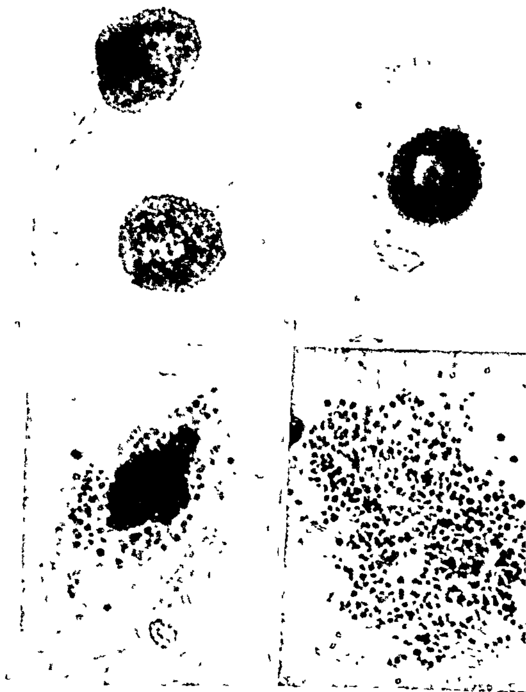


Fig 1

a Normal mast cells with no ejected granules (type A) Aqueous toluidine blue × 1500
b Mast cell with 1 10 ejected granules (type B) Aqueous toluidine blue × 1500
c Mast cell surrounded by granules but with the central part intact (type C) Aqueous toluidine blue, × 1500
d Totally disrupted mast cell with the granules evenly scattered (type D) Aqueous toluidine blue × 1500

The Effect of Hypothermia on the Degranulation of Mast Cells Caused by Turpentine

Sixteen groups of mice were anaesthetized (Table 2) Groups 1–4 were kept at normal body temperature during the experiment, groups 5–8 were cooled to a rectal temperature of 27–27 5° C, groups 9–12 were cooled to 20 5–22 5° C, and groups 13 16 were cooled to 14 5 16° C An intraperitoneal injection of turpentine was given after 1 hour of anaesthesia to the mice in groups 1–4, and after half an hour of hypothermia to the mice in groups 5–16 The concentration of turpentine which was used for the different groups, is shown in Table 2 The mice were killed 45 minutes after the injection of turpentine

The mast cell counts are recorded in Table 2 The counts in groups 1 12 are all from mice which lived during the experiment The count from 1 mouse in group 11 which died under hypothermia, was excluded from calculations All mice in groups 13–16 died under hypothermia Consequently the counts of these groups are not strictly comparable to those of groups 1–12

Degranulation of mast cells was produced by 25 and 100 per cent turpentine It appears from Table 2 that lowering of the body temperature is associated with decrease in degranulation of the mast cells

A two-way analysis of variance was performed for each type of mast cells in groups 1, 2, 5, 6, 9, 10 (25 and 100 per cent turpentine) Arcsin transformation was used for the counts of A and B cells The analyses disclosed significant interaction between concentration of *turpentine and* body temperature for C and D cells, but not for A and B-cells A significant effect of hypothermia was found for mast cells of types A B and D, but not for C cells The distribution of mast cells at 21° C did not differ significantly from that at 27° C

Undiluted turpentine never failed to produce degranulation at body temperatures of 37°, 27° *and* 21° C, *although* the degranulative power decreased with falling temperature Twenty-five per cent turpentine caused degranulation in almost all cases at 37° C, in *half* of the cases at 27°, and in ¼ of the cases at 21° C The threshold concentration of turpentine causing degranulation seemed to be increased when the body temperature decreased This probably explains the interaction which was found between concentration of turpentine and body temperature

At a body temperature *of* 15° C undiluted turpentine caused a slight degranulation of the mast cells in 3 out of 4 mice, while 25 and 5 per cent turpentine did *not cause degranulation* The counts of groups 13 16 were analysed by one way analyses of variance, one analysis for each type of mast cell Arcsin transformation was used for the counts of C and D-cells The distribution of mast cells in groups 14, 15 and 16 was not found to differ significantly The counts of A, C and D-cells in group 13 differed significantly from the corresponding counts of group 14 + 15 + 16 (0 01 > P for A cells, 0 005 > P for C and D-cells)

TABLE 1

Distribution of Mast Cells in the Mesentery of Normothermic Mice 45 Minutes after the Intraperitoneal Injection of Turpentine

Group	Additional treatment	Concentration of turpentine*	Total mast cell count	Types of mast cells in percentages			
				A	B	C	D
1	None	100	300 ± 0	1 8 ± 1 1	2 4 ± 0 9	17 4 ± 5 1	78 3 ± 6 0
2		25	282 ± 19 5	59 3 ± 15 4	15 0 ± 5 3	10 1 ± 5 2	15 6 ± 11 0
3		5	300 ± 0	56 1 ± 37 6	16 9 ± 11 6	14 1 ± 20 7	12 9 ± 25 8
4		0	282 ± 36 5	67 3 ± 14 5	24 5 ± 5 9	6 9 ± 8 1	1 3 ± 1 2
5	Anaesthesia	100	300 ± 0	1 7 ± 1 6	3 1 ± 2 7	16 4 ± 5 1	78 8 ± 8 0
6		25	297 ± 6 0	46 6 ± 12 6	14 4 ± 9 5	21 2 ± 4 7	17 8 ± 8 7
7		5	300 ± 0	82 2 ± 3 2	15 6 ± 2 5	2 1 ± 1 4	0 1 ± 0 2
8		0	300 ± 0	74 7 ± 9 4	18 7 ± 4 9	6 2 ± 4 3	0 4 ± 0 5

The counts recorded in the table are means of 4 observations with standard deviation

* Percentage of turpentine in olive oil

A B C D See text page 3

The Effect of Hypothermia on the Degranulation of Mast Cells Caused by Turpentine

Sixteen groups of mice were anaesthetized (Table 2) Groups 1–4 were kept at normal body temperature during the experiment, groups 5–8 were cooled to a rectal temperature of 27–27 5° C, groups 9–12 were cooled to 20 5–22 5° C, and groups 13 16 were cooled to 14 5–16° C An intraperitoneal injection of turpentine was given after 1 hour of anaesthesia to the mice in groups 1–4, and after half an hour of hypothermia to the mice in groups 5–16 The concentration of turpentine which was used for the different groups, is shown in Table 2 The mice were killed 45 minutes after the injection of turpentine

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Degranulation of mast cells was produced by 25 and 100 per cent turpentine It appears from Table 2 that lowering of the body temperature is associated with decrease in degranulation of the mast cells

A two way analysis of variance was performed for each type of mast cells in groups 1, 2, 5, 6, 9, 10 (25 and 100 per cent turpentine) Arcsin transformation was used for the counts of A and B-cells The analyses disclosed significant interaction between concentration of turpentine and body temperature for C and D cells, but not for A and B cells A significant effect of hypothermia was found for mast cells of types A, B and D, but not for C cells The distribution of mast cells at 21° C did not differ significantly from that at 27° C

Undiluted turpentine never failed to produce degranulation at body temperatures of 37°, 27° and 21° C, although the degranulative power decreased with falling temperature Twenty-five per cent turpentine caused degranulation in almost all cases at 37° C, in half of the cases at 27°, and in ⅓ of the cases at 21° C The threshold concentration of turpentine causing degranulation seemed to be increased when the body temperature decreased This probably explains the interaction which was found between concentration of turpentine and body temperature

At a body temperature of 15° C undiluted turpentine caused a slight degranulation of the mast cells in 3 out of 4 mice, while 25 and 5 per cent turpentine did not cause degranulation The counts of groups 13 16 were analysed by one-way analyses of variance, one analysis for each type of mast cell Arcsin transformation was used for the counts of C and D cells The distribution of mast cells in groups 14, 15 and 16 was not found to differ significantly The counts of A, C and D cells in group 13 differed significantly from the corresponding counts of group 14+15+16 (0 01>P for A-cells, 0 005>P for C and D cells)

TABLE 2

Distribution of Mast Cells in the Mesentery of Anaesthetized Mice at Different Body Temperature—45 Minutes after the Intraperitoneal Injection of Turpentine

Group	Body temperature	Concentration of turpentine*	Total mast cell count	Types of mast cells in percentages			
				A	B	C	D
1	36 5 37 5° C	100	300 ± 0	1 7 ± 1 6	3 1 ± 2 7	16 4 ± 5 1	78 8 ± 8 0
2		25	297 ± 6 0	46 6 ± 12 6	14 4 ± 9 5	21 2 ± 4 7	17 8 ± 8 7
3		5	300 ± 0	82 2 ± 3 2	15 6 ± 2 5	2 1 ± 1 4	0 1 ± 0 2
4		0	300 ± 0	74 7 ± 9 4	18 7 ± 4 9	6 2 ± 4 3	0 4 ± 0 5
5	27 27 5° C	100	300 ± 0	6 6 ± 1 1	6 8 ± 2 2	38 2 ± 5 5	48 4 ± 7 0
6		25	278 ± 23 8	70 8 ± 10 7	11 1 ± 3 1	10 3 ± 6 9	7 8 ± 7 1
7		5	300 ± 0	67 7 ± 28 6	19 7 ± 11 3	9 9 ± 13 9	2 7 ± 4 0
8		0	292 ± 10 4	66 1 ± 14 4	22 7 ± 7 8	10 3 ± 10 2	0 9 ± 1 0
9	20 5 22 5° C	100	276 ± 48 0	12 3 ± 6 0	12 9 ± 4 5	35 8 ± 6 2	38 9 ± 13 3
10		25	288 ± 14 9	59 9 ± 16 8	21 2 ± 9 2	14 6 ± 9 5	4 3 ± 6 9
11		5	300 ± 0	82 7 ± 3 6	15 4 ± 4 1	1 7 ± 0 5	0 2 ± 0 2
12		0	289 ± 18 1	89 1 ± 4 8	8 6 ± 3 8	2 1 ± 1 9	0 3 ± 0 2
13	14 5 16 ° C	100	277 ± 47 0	46 6 ± 21 1	29 9 ± 10 0	18 7 ± 9 3	4 8 ± 4 3
14		25	294 ± 9 4	66 4 ± 11 0	24 7 ± 7 5	8 5 ± 5 3	0 4 ± 0 3
15		5	300 ± 0	85 8 ± 10 7	12 0 ± 8 0	2 0 ± 2 3	0 3 ± 0 5
16		0	294 ± 12 5	67 3 ± 12 0	25 2 ± 7 8	6 9 ± 4 1	0 6 ± 0 7

The counts recorded in the table are means of 4 observations with standard deviation (3 observations in group 11)
A B C D See text page 5
* Percentage of turpentine in olive oil
The mice of groups 13 16 died before the injection of turpentine Groups 1 12 include mice exclusively which remained alive during the experiment

Five per cent turpentine did not cause degranulation at any temperature level At all temperatures the groups treated with 5 per cent turpentine showed about the same distribution of mast cells as the controls treated with olive oil The body temperature did not significantly influence the distribution of mast cells in these groups

The Effect of Hypothermia on the Rate of Degranulation after Injection of Turpentine

This experiment was carried out to find the rate at which the degranulation caused by turpentine, takes place under normothermia and hypothermia Only undiluted turpentine was used, as the former experiment showed that undiluted turpentine caused more uniform changes than lower concentrations

Eight groups of mice were used (Table 3) Groups 1–4 were anaesthetized and kept at normal body temperature Groups 5–8 were anaesthetized and cooled to a rectal temperature of 20 5–22 5° C An intraperitoneal injection of turpentine was given after 1 hour of anaesthesia to the mice in groups 1–4, and after half an hour of hypothermia to the mice in groups 5–8 Groups of normothermic and hypothermic mice were killed 5, 15, 45 and 120 minutes, respectively, after the injection of turpentine One mouse in group 6 died under hypothermia and was excluded from calculations

The mast cell counts are recorded in Table 3 For each type of mast cell a two way analysis of variance was performed, followed by test of all comparisons among means

Extensive degranulation of the mast cells was found in all normothermic mice with the exception of one mouse in group 1 in which the count revealed normal distribution of the mast cells (A-cells 73 per cent, B-cells 24 per cent, C-cells 2 3 per cent, D cells 0 3 per cent)

It will be seen from the table that hypothermia was associated with reduced degranulation of the mast cells The analyses disclosed a significant effect of hypothermia for all cell types (0 025>P for A-cells, 0 005>P for B, C and D cells) No significant interaction between hypothermia and the duration of exposure to turpentine was found

At normal body temperature the degranulation of mast cells was nearly maximal 5 minutes after the injection of turpentine (except in one case in which no degranulation was found) Consequently an increase in exposure to turpentine from 5 to 120 minutes did not significantly increase the degranulation of the mast cells

Under hypothermia a moderate degranulation of the mast cells was found 5 minutes after injection of turpentine, and an increase in exposure to turpentine from 5 to 120 minutes was associated [illegible]

[illegible]

[illegible]ranulation found in hypothermic mice

TABLE 3

Distribution of Mast Cells in the Mesentery of Anaesthetized Mice at Different Intervals after the Intraperitoneal Injection of Undiluted Turpentine

Group	Body temperature	Exposure to turpentine in minutes	Total mast cell count	Types of mast cells in percentages			
				A	B	C	D
1	36.5–37.5° C	5	300 ± 0	18.8 ± 36.3	7.3 ± 11.2	22.7 ± 15.7	51.2 ± 34.7
2		15	300 ± 0	0.8 ± 1.0	2.2 ± 0.6	27.8 ± 7.4	69.2 ± 8.2
3		45	300 ± 0	1.7 ± 1.6	3.1 ± 2.7	16.4 ± 5.1	78.8 ± 8.0
4		120	300 ± 0	2.8 ± 2.6	3.9 ± 2.1	17.0 ± 7.3	76.3 ± 8.3
5	20.5–22.5° C	5	300 ± 0	21.1 ± 13.6	19.5 ± 2.2	36.3 ± 4.8	23.1 ± 7.2
6		15	300 ± 0	16.7 ± 8.1	13.3 ± 4.2	33.0 ± 4.8	37.0 ± 11.4
7		45	276 ± 48	12.3 ± 6.0	12.9 ± 4.5	35.8 ± 6.2	38.9 ± 13.3
8		120	300 ± 0	6.5 ± 3.2	6.2 ± 2.6	30.5 ± 9.2	56.8 ± 9.2

The counts recorded in the table are means of 4 observations (3 observations in group 6) with standard deviation

A B C D See text page 7

120 minutes after the injection of turpentine was similar to that found after 5 minutes in normothermic mice

DISCUSSION

The present investigation showed that in normal mice the intraperitoneal injection of undiluted turpentine caused pronounced degranulation 25 per cent turpentine caused moderate degranulation while 5 per cent turpentine usually did not cause any degranulation of the mast cells in the mesentery

The degranulative action of turpentine on the mast cells was not influenced by pentobarbital anaesthesia when the body temperature was kept normal

Hypothermia was associated with partial inhibition of the degranulation caused by turpentine The degranulation decreased proportionally with decrease in body temperature The degranulation proceeded much more slowly under hypothermia than under normothermia Thus the degree of degranulation found 2 hours after injection of turpentine in hypothermic mice (21° C) was similar to that found after 5 minutes in normothermic mice

It is well estblished that turpentine causes degranulation of mast cells *Spector & Willoughby* (1959) found that injection of turpentine into the pleural space of rats caused pleurisy with degranulation of the mast cells *Gustafsson & Cronberg* (1957) showed that intraperitoneal injection of turpentine in rats caused pronounced degranulation of the mast cells in the mesentery The degranulation caused by turpentine was similar to that caused by high concentrations of the histamine releaser compound 48.80

In vitro studies have shown that the action of 48 80 on rat ma[illegible]

[illegible] mast cells decreased when the temperature was lowered *Uvnas & Thon* (1961) studying the histamine releasing action of 48 80 on mast cells isolated from rat peritoneal cavity found that the histamine release diminished with falling incubation temperature

Th[illegible]

a su[illegible]

(19[illegible] [illegible] ast cells by activating a lytic enzyme attached to the cell membrane Whether or not turpentine degranulates mast cells through the same mechanism as 48 80 is not known It is common knowledge however that the rate of enzyme catalysed reactions is temperature dependent If turpentine degranulates mast cells through an enzyme mechanism the delay in degranulation found under hypothermia might in part be explained by reduced enzymatic activity under hypothermia

TABLE 3

Distribution of Mast Cells in the Mesentery of Anaesthetized Mice at Different Intervals after the Intraperitoneal Injection of Undiluted Turpentine

Group	Body temperature	Exposure to turpentine *in minutes*	Total mast cell *count*	Types of mast cells in percentages			
				A	B	C	D
1	36 5–37 5° C	5	300 ± 0	18 8 ± 36 3	7 3 ± 11 2	22 7 ± 15 7	51 2 ± 34 7
2		15	300 ± 0	0 8 ± 1 0	2 2 ± 0 6	27 8 ± 7 4	69 2 ± 8 2
3		45	300 ± 0	1 7 ± 1 6	3 1 ± 2 7	16 4 ± 5 1	78 8 ± 8 0
4		120	300 ± 0	2 8 ± 2 6	3 9 ± 2 1	17 0 ± 7 3	76 3 ± 8 3
5	20 5–22 5° C	5	300 ± 0	21 1 ± 13 6	19 5 ± 2 2	36 3 ± 4 8	23 1 ± 7 2
6		15	300 ± 0	16 7 ± 8 1	13 3 ± 4 2	33 0 ± 4 8	37 0 ± 11 4
7		45	276 ± 48	12 3 ± 6 0	12 9 ± 4 5	35 8 ± 6 2	38 9 ± 13 3
8		120	300 ± 0	6 5 ± 3 2	6 2 ± 2 6	30 5 ± 9 2	56 8 ± 9 2

The counts recorded in the table are means of 4 observations (3 observations in group 6) with standard deviation
A, B, C, D See text page 7

Pentobarbital anaesthesia did not alter the degranulation caused by turpentine when the body temperature was kept normal

Hypthermia was associated with reduced degranulation of mast cells after injection of turpentine The degranulation diminished with falling body temperature In hypothermic mice the degranulation proceeded more slowly than in normothermic mice In mice cooled to 20 5–22 5° C the degree of degranulation found 2 hours after injection of turpentine, was similar to that found after 5 minutes in normothermic mice

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After intraperitoneal injection turpentine has to pass through the peritoneum to reach the mast cells in the mesentery Turpentine is highly soluble in lipoids and nearly insoluble in water, and the molecular size is moderate (*Fehling* 1905) Therefore the ability of turpentine to penetrate cell membranes can be expected to be great at normal body temperature (*Giese* 1962) The observation that the degranulation of mast cells occurs within a short time after the injection of turpentine at normal body temperature is in accordance with this expectation

The permeability of cell membranes is temperature dependent The rate at which molecules move through a cell membrane in response to a concentration gradient, decreases when the temperature is lowered However, a considerable difference in temperature coefficient is found for the passage of different types of molecules through cell membranes (*Bělehradec* 1935, *Davson & Danielli* 1952, *Rogers & McElroy* 1958) The temperature coefficient for turpentine is not known However, the passage of turpentine through the peritoneum to the mesenteric mast cells, is probably retarded under hypothermia This may in part explain the delay in degranulation of mast cells found under hypothermia

The present experiment shows that the degranulation of mast cells in the mesentery of mice, caused by intraperitoneal injection of turpentine, was partially inhibited under hypothermia However, the experiment fail to indicate whether the inhibition were due to reduced permeability of the peritoneum to turpentine, or to increased resistance of the mast cells to the degranulative action of turpentine, or both

Mast cells in rats normally contain histamine and 5-HT (*Riley & West* 1953, *Benditt, Wong, Arase & Roeper* 1955) and disrupture of the mast cells is associated with release of these substances (*Riley & West* 1955b *Bhattacharya & Lewis* 1956, *Rowley & Benditt* 1956, *Garcia Arocha* 1961) Considerable evidence has also been provided that the mast cells in mice contain histamine (*West* 1955, *Riley & West* 1955a) and 5 HT (*Parratt & West* 1957, *Furth, Hagen & Hirsch* 1957, *Sjoerdsma, Waalkes & Weissbach* 1957, *Giarman, Potter & Day* 1960 *Coupland & Riley* 1960) Since the degranulation of mesenteric mast cells induced by turpentine in mice is partially inhibited under hypothermia the release of histamine and 5-HT can be expected to be reduced under hypothermia And reduced release of these substances, which are both vasoactive may be one of the causes to the reduced oedema formation in turpentine inflammation in hypothermic mice

SUMMARY

Acute peritonitis was produced by intraperitoneal injection of turpentine into mice Mesenteric spreads were used in a study of the mast cells

In normal mice turpentine caused extensive degranulation of the mast cells in the mesentery

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STUDIES IN HYPOTHERMIA

5 *The Rôle of Reduced Release of Chemical Mediators in Turpentine Inflammation in Hypothermic Mice*

By

KNUT SVANES

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It has been shown previously (*Svanes* 1964) that the initial oedema formation in acute turpentine inflammation in the skin of mice is reduced under hypothermia

Spector & Willoughby (1959) found that the increased capillary permeability in turpentine pleurisy in rats was initiated by local release of histamine No direct action of turpentine on the capillary walls was found

The mediators of the oedema caused by turpentine in the skin of mice are not known However, considerable evidence has been provided that the mast cells in mice contain histamine and 5-hydroxytryptamine (*West* 1955, *Riley & West* 1955, *Parratt & West* 1957, *Furth, Hagen & Hirsch* 1957, *Sjoerdsma, Waalkes & Weissbach* 1957, *Giarman, Potter & Day* 1960, *Coupland & Riley* 1960) Histamine and 5 HT are vaso-active substances which cause increased capillary permeability when injected into the skin of mice Turpentine exerts a strong degranulative action on the mast cells in normothermic mice This indicates that 5-HT and histamine might be mediators of the initial oedema caused by turpentine in the skin of mice Since the degranulation of mast cells in turpentine inflammation is partially inhibited under hypothermia (*Svanes* 1965), the release of 5 HT and histamine is probably reduced under hypothermia

The present investigation was designed to discover whether reduced release of mediators might account for the reduced oedema formation in turpentine inflammation under hypothermia

Three experiments were performed 1) to test the power and specificity of a histamine antagonist (mepyramine) and a serotonine antagonist (methysergid), 2) to determine the nature of the mediators in the initial phase of turpentine inflammation by means of antagonists,

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It has been shown previously (*Svanes* 1964) that the initial oedema formation in acute turpentine inflammation in the skin of mice is reduced under hypothermia

Spector & Willoughby (1959) found that the increased capillary permeability in turpentine pleurisy in rats was initiated by local release of histamine No direct action of turpentine on the capillary walls was found

The mediators of the oedema caused by turpentine in the skin of mice are not known However, considerable evidence has been provided that the mast cells in mice contain histamine and 5 hydroxytryptamine (*West* 1955, *Riley & West* 1955, *Parratt & West* 1957, *Furth, Hagen & Hirsch* 1957, *Sjoerdsma, Waalkes & Weissbach* 1957, *Giarman, Potter & Day* 1960, *Coupland & Riley* 1960) Histamine and 5 HT are vaso-active substances which cause increased capillary permeability when injected into the skin of mice Turpentine exerts a strong degranulative action on the mast cells in normothermic mice This indicates that 5 HT and histamine might be mediators of the initial oedema caused by turpentine in the skin of mice Since the degranulation of mast cells in turpentine inflammation is partially inhibited under hypothermia (*Svanes* 1965), the release of 5 HT and histamine is probably reduced under hypothermia

The present investigation was designed to discover whether reduced release of mediators might account for the reduced oedema formation in turpentine inflammation under hypothermia

Three experiments were performed 1) to test the power and specificity of a histamine antagonist (mepyramine) and a serotonine antagonist (methysergid), 2) to determine the nature of the mediators in the initial phase of turpentine inflammation by means of antagonists,

Supported by grants from "Dr Alexander Malthes Legat and 'Dr Nils Henrichsens og hustru Anna Henrichsens legat

3) to show whether the oedema produced by injection of the mediators would be influenced by hypothermia in the same way as the oedema produced by turpentine

MATERIALS AND METHODS

Animals Male albino mice 6 12 weeks old were used

Turpentine inflammation was produced by placing 2 drops of turpentine oil on the abdominal skin twice at an interval of 15 minutes On the preceding day the abdomen had been shaved by a machine clipper

Histamine was employed as its dihydrochloride and used as 50 and 500 μg/ml solutions in isotonic saline of which 0 04 ml was injected subcutaneously into the abdominal wall of the mice

5 hydroxytryptamine (5 HT) was employed as serotonin creatinine sulphate supplied by Messrs L Light & Co and used as 25 and 100 μg/ml solutions in isotonic saline Of these 0 04 ml was injected subcutaneously into the abdominal wall of the mice

Histamine antagonist Mepyramine maleate (Anthisan) supplied by Messrs May & Baker, was used as 0 25 per cent solution w/v in isotonic saline and given intravenously in doses of 2 10 mg/kg body weight 15 20 minutes before the application of turpentine histamine and 5 HT

Serotonine antagonist 1 methyl d lysergic acid butanolamide (methysergid bimaleinat) supplied by Messrs Sandoz was used as 0 005 0 05 per cent solutions w/v in isotonic saline and applied by subcutaneous injection to the back in doses of 0 1 1 0 mg/kg body weight 30 minutes before the application of turpentine histamine and 5 HT

After treatment with mepyramine and methysergid the body temperature of the mice showed a slight tendency to fall at an ambient temperature of about 22° C To counteract this the mice were gently warmed under a light bulb

Oedema and dye leakage The local oedema caused by turpentine histamine and 5 HT was estimated by the trypan blue technique 3 1 ml/kg body weight of a 1 per cent solution of trypan blue was given intravenously just prior to the treatment with turpentine histamine and 5 HT The mice were killed 30 minutes later Subcutaneous injection of histamine and 5 HT caused local accumulation of dye in the skin and the adjacent musculature while epicutaneous application of turpentine caused dye accumulation only in the skin Standard samples including all layers of the abdominal wall were therefore removed from the mice treated with histamine and 5 HT (Tables 1 and 3) while samples of skin alone were usually removed from the turpentine treated mice (Table 2) The content of trypan blue in the samples was determined by extraction and measurement in a spectrophotometer (*Judah & Willoughby* 1962 *Svanes* 1964) Trypan blue was estimated at 600 mμ in cells with a 1 cm light path A final volume of 3 5 ml was used for samples consisting of skin alone 4 ml was used for samples including all layers of the abdominal wall

In this paper dye leakage is defined as the amount of trypan blue which corresponds to the local oedema caused by histamine 5 HT and turpentine The dye leakage was estimated as the difference between the amount of dye extracted from samples of mice locally treated with turpentine histamine and 5 HT and the amount of dye extracted from samples of control mice not treated with these substances

Anaesthesia and *hypothermia* The technique of anaesthesia and hypothermia has been described in detail previously (*Svanes* 1964) Sodium pentobarbital was used for anaesthesia The body temperature remained normal when the anaesthetized mice were kept in a thermostat at 32 34° C Hypothermia was induced by cooling anaesthetized mice in a refrigerator at 4° C for 8 9 minutes When the mice subsequently were kept in a thermostat at 20° C throughout the experiment the rectal temperature remained at 20 5 22° C

RESULTS

The Effect of Mepyramine and Methysergid on the Oedema Produced by Histamine and 5-hydroxytryptamine

Eighty six mice about 10 weeks old and weighing 33.5 ± 2.3 g were used. They were distributed randomly in 21 groups (see Table 1). The mice were given an intravenous injection of trypan blue, and immediately after this histamine was injected subcutaneously into the mice in groups 1–10 and 5-HT into the mice in groups 11–20. Groups 3–6 and 13–16 were pretreated with mepyramine and groups 7–10 and 17–20 were pretreated with methysergid. The dye leakage was determined as described above.

TABLE 1

The Effect of Mepyramine and Methysergid on the Leakage of Circulating Trypan Blue Induced in Mice by Subcutaneous Injection of Histamine and 5-Hydroxytryptamine (5-HT)

Group	Permeability factor	Inhibitor	Extracted trypan blue	Dye leakage§
1	Histamine 500 µg/ml	0	0.258 ± 0.030	0.215 ± 0.030
2	Histamine 50 µg/ml	0	0.129 ± 0.054	0.086 ± 0.054
3	Histamine 500 µg/ml	Mepyramine 2 mg/kg	0.071 ± 0.035	0.028 ± 0.035
4	Histamine 500 µg/ml	Mepyramine 10 mg/kg	0.048 ± 0.014	0.005 ± 0.014
5	Histamine 50 µg/ml	Mepyramine 2 mg/kg	0.045 ± 0.019	0.002 ± 0.019
6	Histamine 50 µg/ml	Mepyramine 10 mg/kg	0.029 ± 0.004	−0.014 ± 0.004
7	Histamine 500 µg/ml	Methysergid 0.1 mg/kg	0.213 ± 0.058	0.170 ± 0.058
8	Histamine 500 µg/ml	Methysergid 1.0 mg/kg	0.106 ± 0.021	0.063 ± 0.021
9	Histamine 50 µg/ml	Methysergid 0.1 mg/kg	0.116 ± 0.020	0.073 ± 0.020
10	Histamine 50 µg/ml	Methysergid 1.0 mg/kg	0.137 ± 0.031	0.094 ± 0.031
11	5-HT 100 µg/ml	0	0.251 ± 0.044	0.208 ± 0.044
12	5-HT 25 µg/ml	0	0.186 ± 0.028	0.143 ± 0.028
13	5-HT 100 µg/ml	Mepyramine 2 mg/kg	0.179 ± 0.078	0.136 ± 0.078
14	5-HT 100 µg/ml	Mepyramine 10 mg/kg	0.133 ± 0.029	0.090 ± 0.029
15	5-HT 25 µg/ml	Mepyramine 2 mg/kg	0.151 ± 0.024	0.108 ± 0.024
16	5-HT 25 µg/ml	Mepyramine 10 mg/kg	0.148 ± 0.045	0.105 ± 0.045
17	5-HT 100 µg/ml	Methysergid 0.1 mg/kg	0.061 ± 0.007	0.018 ± 0.007
18	5-HT 100 µg/ml	Methysergid 1.0 mg/kg	0.045 ± 0.012	0.002 ± 0.012
19	5-HT 25 µg/ml	Methysergid 0.1 mg/kg	0.041 ± 0.007	−0.002 ± 0.007
20	5-HT 25 µg/ml	Methysergid 1.0 mg/kg	0.040 ± 0.010	−0.003 ± 0.010
21	Saline	0	0.043 ± 0.011	

Mean of 4 observations (6 in group 21) with standard deviation. The amount of trypan blue is expressed as the extinction values found on spectrophotometry. 0.067 = 5 µg.

§ Dye leakage = The difference between the extracted trypan blue values of groups 1–20 and the mean value of group 21.

The results are recorded in Table 1 and Figures 1 and 2. In normal mice the injection of 50 and 500 µg/ml histamine caused pronounced dye leakage. In mice pretreated with 2 and 10 mg/kg mepyramine the injection of 50 µg/ml histamine did not cause dye leakage, while 500 µg/ml histamine caused only minimal leakage. Pretreatment with 0.1

3) to show whether the oedema produced by injection of the mediators would be influenced by hypothermia in the same way as the oedema produced by turpentine

MATERIALS AND METHODS

Animals Male albino mice 6 12 weeks old were used

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Serotonine antagonist 1 methyl d lysergic acid butanolamide (methysergid bimaleinat) supplied by Messrs Sandoz was used as 0 005 0 05 per cent solutions w/v in isotonic saline and applied by subcutaneous injection to the back in doses of 0 1–1 0 mg/kg body weight 30 minutes before the application of turpentine histamine and 5 HT

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Oedema and *dye leakage* The local oedema caused by turpentine histamine and 5 HT was estimated by the trypan blue technique 3 1 ml/kg body weight of a 1 per cent solution of trypan blue was given intravenously just prior to the treatment with turpentine histamine and 5 HT The mice were killed 30 minutes later Subcutaneous injection of histamine and 5 HT caused local accumulation of dye in the skin and the adjacent musculature while epicutaneous application of turpentine caused dye accumulation only in the skin Standard samples including all layers of the abdominal wall were therefore removed from the mice treated with histamine and 5 HT (Tables 1 and 3) while samples of skin alone were usually removed from the turpentine treated mice (Table 2) The content of trypan blue in the samples was determined by extraction and measurement in a spectrophotometer (*Judah & Willoughby* 1962 *Stanes* 1964) Trypan blue was estimated at 600 mµ in cells with a 1 cm light path A final volume of 3 5 ml was used for samples consisting of skin alone 4 ml was used for *samples including all layers of the abdominal wall*

In this paper dye leakage is defined as the amount of trypan blue which corresponds to the local oedema caused by histamine 5 HT and turpentine The dye leakage was estimated as the difference between the amount of dye extracted from samples of mice locally treated with turpentine histamine and 5 HT and the amount

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The technique of anaesthesia and hypothermia has

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and 1 mg/kg methysergid did not seem to influence the dye leakage caused by 50 µg/ml histamine, while the dye leakage caused by 500 µg/ml was slightly reduced by 0.1 mg/kg methysergid and greatly reduced by 1 mg/kg methysergid.

The data of groups 1, 2, 7, 8, 9, 10 in Table 1 were analysed by a two-way analysis of variance. A significant effect of methysergid was found ($0.01 > P$), and significant interaction between the doses of histamine and methysergid ($0.005 > P$). A test of all comparisons among means revealed no significant differences between groups 2, 9 and 10, no difference between groups 1 and 7, but a significant difference between groups 1 and 8 ($0.05 > P$).

In normal mice the injection of 25 and 100 µg/ml 5-HT caused pronounced dye leakage. Pretreatment with 2 and 10 mg/kg mepyramine moderately reduced the dye leakage after injection of 25 and 100 µg/ml 5-HT. In mice pretreated with 0.1 and 1 mg/kg methysergid the injection of 25 µg/ml 5-HT did not cause dye leakage, while 100 µg/ml 5-HT only caused a minimal dye leakage.

The data of groups 11–16 were analysed by a two-way analysis of variance. The analysis disclosed a significant effect of mepyramine ($0.005 > P$), but no significant difference between the effect of 2 and 10 mg/kg of mepyramine. No interaction between the doses of 5-HT and mepyramine was found.

TABLE 2

The Effect of Mepyramine and Methysergid on the Leakage of Circulating Trypan Blue into the Skin of Mice after Local Application of Turpentine

Group	Turpentine	Inhibitor	Number of mice	Extracted trypan blue*	Dye leakage µg
1	+	0	16	0.207 ± 0.103	0.182 ± 0.103
2	+	Mepyramine 2 mg/kg	8	0.163 ± 0.073	0.138 ± 0.073
3	+	Mepyramine 5 mg/kg	8	0.180 ± 0.117	0.155 ± 0.117
4	+	Mepyramine 10 mg/kg	8	0.123 ± 0.034	0.098 ± 0.034
5	+	Methysergid 0.1 mg/kg	8	0.131 ± 0.071	0.106 ± 0.071
6	+	Methysergid 0.5 mg/kg	8	0.104 ± 0.053	0.079 ± 0.053
7	+	Methysergid 1.0 mg/kg	8	0.123 ± 0.067	0.098 ± 0.067
8	+	Mepyramine 2 mg/kg + Methysergid 0.1 mg/kg	8	0.075 ± 0.024	0.050 ± 0.024
9	+	Mepyramine 5 mg/kg + Methysergid 0.5 mg/kg	8	0.066 ± 0.019	0.041 ± 0.019
10	+	Mepyramine 10 mg/kg + Methysergid 1.0 mg/kg	8	0.054 ± 0.013	0.029 ± 0.013
11	0	0	8	0.025 ± 0.007	

* [illegible] ean value of group 11

The values shown in the table are means with standard deviation

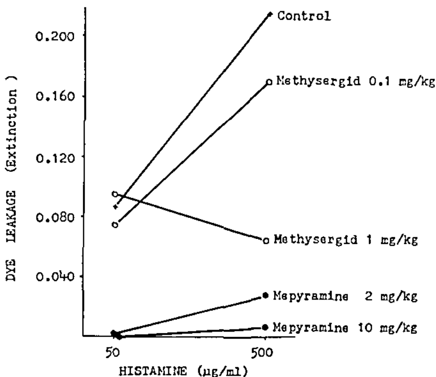


Fig 1.
The effect of methysergid and mepyramine on the leakage of circulataing trypan blue induced in mice by subcutaneous injection of histamine.

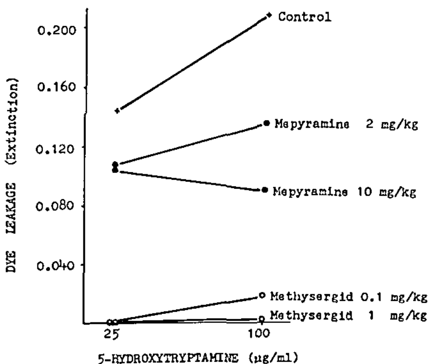


Fig 2
The effect of methysergid and mepyramine on the leakage of circulataing trypan blue induced in mice by subcutaneous injection of 5-hydroxytryptamine

decrease in the dye leakage (0 005>P), but no significant differences were found between the effect of 0 1, 0 5 and 1 0 mg kg of methysergid After treatment with mepyramine and methysergid in combination (groups 8, 9 and 10 considered together) the dye leakage was greatly reduced The effect of combined treatment differed significantly (0 005>P) from that of methysergid alone No significant differences were found between the dye leakage in groups 8, 9 and 10

Pretreatment with mepyramine (groups 2, 3 and 4 considered collectively) reduced the dye leakage by 29 per cent, methysergid (groups 5, 6 and 7 considered together) reduced it by 48 per cent, and mepyramine and methysergid in combination (groups 8, 9 and 10 considered together) reduced the dye leakage by 78 per cent The inhibition caused by simultaneous administration of mepyramine and methysergid was about equal to the sum of the inhibitions caused by mepyramine and methysergid by themseelves

The Effect of Anaesthesia and Hypothermia on the Oedema Produced by Turpentine, Histamine and 5 Hydroxytryptamine

In this experiment 120 mice, 6–8 weeks old and weighing 29 9 ± 1 7 g, were used They were distributed at random in 15 groups (see Table 3) Five groups (1, 4, 7, 10, 13) were kept under normal conditions during the experiment, five groups (2, 5, 8, 11, 14) were anaesthetized and kept at normal body temperature, and 5 groups (3, 6, 9, 12, 15) were anaesthetized and subjected to hypothermia Apart from the treatment just described the corresponding normal, anaesthetized and hypothermic groups were given the same treatment An intravenous injection of trypan blue was given after 1 hour of anaesthesia to the mice in groups 2 5, 8, 11, 14 and after ½ hour of hypothermia to the mice in groups 3 6 9, 12, 15 Immediately after the injection of trypan blue the mice in groups 1–3 were treated with turpentine on the abdominal skin, groups 4 6 were given a subcutaneous injection of histamine (500 µg ml), groups 7–9 were given a subcutaneous injection of 5 HT (100 µg ml), and groups 10–12 were given a subcutaneous injection of 0 04 ml isotonic saline to the abdomen Groups 13–15 were not treated locally on the abdomen

The results are recorded in Table 3 The dye leakage values of groups 1 9 were analysed statistically by a one way analysis of variance followed by a test of all comparisons among means which gives the differences significant at the 5 per cent level Group 2 was not found to differ significantly from group 1 [illegible]
group 3 [illegible]
8 d[illegible]
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[illegible] normothermic groups 2, 5 and [illegible], or between the hypothermic groups 3, 6 and 9

Compared to the normal values, anaesthesia reduced the dye leakage

The Effect of Mepyramine and Methysergid on the Oedema Produced by Turpentine.

Ninety-six mice, 11–12 weeks old and weighing 32 8 ± 2 5 g, were used. They were distributed randomly in 11 groups (see Table 2). The mice were given an intravenous injection of trypan blue, and immediately afterwards the mice of groups 1–10 were treated locally with turpentine. Groups 2–4 were pretreated with mepyramine, groups 5–7 with methysergid, and groups 8–10 with mepyramine and methysergid in combination. The dye leakage was determined as described above.

TABLE 3

The Effect of Anaesthesia and Hypothermia on the Leakage of Circulating Trypan Blue Induced in Mice by Local Treatment with Turpentine, Histamine and 5-Hydroxytryptamine (5-HT)

Group	General state	Permeability factor	Number of mice	Extracted tryp in blue*	Dye leakage§
1	N	Turpentine	10	0 135 ± 0 066	0 093 ± 0 066
2	A	Turpentine	10	0 121 ± 0 089	0 092 ± 0 089
3	H	Turpentine	10	0 048 ± 0 031	0 020 ± 0 031
4	N	Histamine	10	0 295 ± 0 049	0 247 ± 0 049
5	A	Histamine	10	0 124 ± 0 041	0 087 ± 0 041
6	H	Histamine	10	0 065 ± 0 016	0 036 ± 0 016
7	N	5-HT	10	0 281 ± 0 065	0 233 ± 0 065
8	A	5-HT	10	0 147 ± 0 063	0 110 ± 0 063
9	H	5 HT	10	0 044 ± 0 021	0 015 ± 0 021
10	N	Saline	5	0 048 ± 0 018	
11	A	Saline	5	0 037 ± 0 010	
12	H	Saline	5	0 029 ± 0 011	
13	N	0	5	0 042 ± 0 004	
14	A	0	5	0 029 ± 0 005	
15	H	0	5	0 028 ± 0 012	

N = *Normal conditions*
A = Barbiturate anaesthesia, normothermia
H = Barbiturate anaesthesia hypothermia
* The amount of trypan blue is expressed as the extinction values found on spectrophotometry, 0 067 E = 5 μg
§ See text p 19
The values shown in the table are means with standard deviation

The results are recorded in Table 2 The trypan blue data of groups 1–10 were analysed by a one-way analysis of variance, after Log_{10} transformation Turpentine usually caused a great dye leakage in normal mice. Pretreatment with mepyramine (groups 2, 3 and 4 considered together) did not significantly reduce the dye leakage caused by turpentine, and no significant differences were found between the effect of 2, 5 and 10 mg/kg of mepyramine Pretreatment with methysergid (groups 5, 6 and 7 considered together) caused a highly significant

the results of the present investigation However, the results are not strictly comparable *Spector & Willoughby* used rats intradermal injection of 5 HT and visual estimation of the dye leakage *Halpern et al* used mice intradermal injection of 5 HT in low doses and visual estimation of the dye leakage while in the present experiment 5 HT was given subcutaneously in rather great doses and the dye leakage was assessed by an objective quantitative method The visual estimation of the dye leakage is difficult and inaccurate at least when used to judge the dye leakage occurring after subcutaneous injection of vaso active substances into mice It is suggested that the discrepancy in results just quoted in part at least is due to different methods of estimating the dye leakage

The present experiment showed that methysergid was a powerful antagonist of 5 HT This is in accordance with the results of *Fanchamps et al* (1960) These authors also concluded that metysergid was a highly specific antagonist of 5 HT In the present experiment this was found to hold true when methysergid was used in moderate doses Thus 0 1 mg kg methysergid did not significantly alter the dye leakage caused by 50 and 500 µg ml histamine On the other hand 1 mg kg methysergid did not alter the dye leakage caused by 50 µg ml histamine but greatly reduced the leakage normally caused by 500 µg ml histamine It is possible that this may have been due to a general toxic effect caused by simultaneous administration of great doses of methysergid and histamine

In the present experiment 50 µg ml 5 HT (expressed as base) caused about the same dye leakage as 300 µg ml histamine (base) Consequently the oedema producing activity of 5 HT was found to be about 6 times greater than that of histamine This differs considerably from the result obtained by *Halpern et al* (1963) showing that in mice the capillary permeability increasing power of 5 HT was about 100 150 times greater than that of histamine

The dye leakage caused by turpentine was slightly reduced after pretreatment with mepyramine moderately reduced after treatment with methysergid and greatly reduced after treatment with mepyramine and methysergid in combination This result suggests that 5 HT and histamine might be mediators of the increased capillary permeability caused by turpentine Treatment with 1 mg/kg methysergid abolished the dye leakage normally caused by 100 µg ml 5 HT, and 10 mg kg mepyramine nearly abolished the leakage normally caused by 500 µg ml histamine The dye leakage caused by turpentine was smaller than that caused by 5 HT and histamine in the doses just mentioned Consequently if 5 HT were the only mediator one would not expect any dye leakage after application of turpentine to mice pretreated with 1 mg kg methysergid The same consideration holds for 10 mg kg mepyramine if histamine were the only mediator Therefore both 5 HT and histamine seem to be mediators of the increased capillary perme

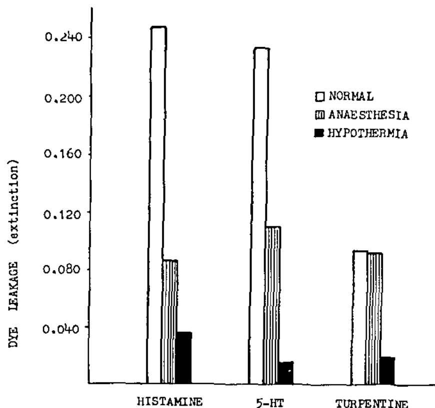


Fig 3

The effect of anaesthesia and hypothermia on the leakage of circulating trypan blue induced in mice by local treatment with turpentine, histamine and 5 HT

caused by turpentine, histamine and 5 HT by 2 per cent, 65 per cent and 53 per cent, respectively Compared to the anaesthetized normothermic values, hypothermia reduced the dye leakage caused by turpentine, histamine and 5 HT by 79 per cent, 58 per cent, and 86 per cent, respectively (see Fig 3)

DISCUSSION

The present experiment showed that mepyramine had a strong antihistamine action However, in doses of 2 and 10 mg/kg it also reduced considerably the dye leakage caused by local injection of 5 HT (25 and 100 μg/ml) *Spector & Willoughby* (1963) tested the effect of mepyramine on the increase in capillary permeability normally caused in the rat by intracutaneous injection of 5-HT (2–10 μg/ml) and some other vaso active substances, and found that mepyramine in doses up to 50 mg/kg was devoid of inhibitory action against any substances other than histamine *Halpern, Neveu & Spector* (1963) found that mepyramine in doses up to 25 mg/kg was without action on the increase in capillary permeability caused by intracutaneous injection of 5-HT (0 0012 μg base) in mice

The results of *Spector & Willoughby* and *Halpern et al* disagree with

after pretreatment with 0 1 and 1 mg kg methysergid (antagonist of serotonine) and greatly although not completely reduced after pre treatment with mepyramine and methysergid in combination The result was found to support the hypothesis that the initial oedema produced by turpentine in the skin of mice was mediated largely through the release of 5 HT and histamine

In mice cooled to a body temperature of 20 5 22 5° C only slight oedema occurred after application of turpentine

Subcutaneous injection of both 100 μg ml 5 HT and of 500 μg/ml histamine caused great local oedema in normothermic mice In hypo thermic mice only slight oedema occurred after the same treatment Oedema caused by 5 HT and histamine and oedema caused by turpen tine were almost equally reduced by hypothermia It was concluded that the scanty oedema formation after application of turpentine to hypothermic mice could not be accounted for only by the reduced release of 5 HT and histamine

In normothermic mice barbiturate anaesthesia greatly reduced the oedema caused by 5 HT and histamine, but not the oedema caused by turpentine

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ability caused by turpentine This would have been proved if mepyramine and methysergid had had no inhibitory action on any vascular permeability factor other than 5-HT and histamine *Sepctor & Willoughby* (1963) found that this was the case for mepyramine Methysergid is believed to be a specific antagonist of 5-HT However, its effect on vascular permeability factors other than 5-HT and histamine does not appear to have been tested It is therefore concluded that the present experiment provides considerable evidence that 5-HT and histamine are the main mediators of the initial increase in capillary permeability caused by turpentine, although this has not been proved directly

This is also in accordance with the fact that turpentine degranulates the mast cells and causes release of 5-HT and histamine

Pretreatment with mepyramine and methysergid in combination did not completely inhibit the dye leakage after application of turpentine This suggests that the increased capillary permeability caused by turpentine, might be mediated in part by vaso-active substances other than histamine and 5-HT

It has been shown previously (*Svanes* 1964) that the initial oedema formation in turpentine inflammation in the skin of mice, was reduced under hypothermia Further it has been shown (*Svanes* 1965) that the degranulation of mast cells in turpentine peritonitis in mice was partially inhibited under hypothermia The release of 5-HT and histamine in turpentine inflammation can therefore be expected to be reduced under hypothermia No doubt the oedema formation is influenced by the amount of mediators released (histamine and 5-HT) However, the present investigation shows that hypothermia is associated with reduced local oedema formation after injection of histamine and 5-HT as well as after application of turpentine The oedema caused by histamine and 5-HT was reduced to a degree similar to that caused by turpentine Consequently the reduced release of histamine and 5 HT cannot be the main cause of the reduced oedema formation in turpentine inflammation under hypothermia Since the oedema caused by turpentine, histamine and 5 HT was similarly *influenced by hypothermia, a common cause* for the reduced oedema has to be discovered

Barbiturate anaesthesia did not significantly alter the oedema formation caused by turpentine, while the oedema caused by histamine and 5-HT was *greatly reduced under anaesthesia at normal* body temperature Further investigation is required to explain this difference in effect of anaesthesia on the oedema producing activity of turpentine histamine and 5-HT

SUMMARY

Normally application of turpentine to the ventral skin of mice causes oedema of the skin

The oedema was slightly diminished in mice pretreated with 2 and 10 mg/kg mepyramine (antagonist of histamine), considerably reduced

after pretreatment with 0 1 and 1 mg kg methysergid (antagonist of serotonine) and greatly although not completely reduced after pre treatment with mepyramine and methysergid in combination The result was found to support the hypothesis that the initial oedema produced by turpentine in the skin of mice was mediated largely through the release of 5 HT and histamine

In mice cooled to a body temperature of 20 5–22 5° C only slight oedema occurred after application of turpentine

Subcutaneous injection of both 100 μg ml 5 HT and of 500 μg ml histamine caused great local oedema in normothermic mice In hypo thermic mice only slight oedema occurred after the same treatment Oedema caused by 5 HT and histamine and oedema caused by turpen tine were almost equally reduced by hypothermia It was concluded that the scanty oedema formation after application of turpentine to hypothermic mice could not be accounted for only by the reduced release of 5 HT and histamine

In normothermic mice barbiturate anaesthesia greatly reduced the oedema caused by 5 HT and histamine but not the oedema caused by turpentine

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Med Department B (Head Professor O Storstein), Institute of General and Experimental Pathology (Head Professor O H Iversen) and Institute of Pathological Anatomy (Head Professor O Torgersen), Rikshospitalet, Oslo Norway

TOTAL JEJUNAL VILLOUS ATROPHY IN SECONDARY STEATORRHOEA

By

EGIL GJONE, JOHANNES MYREN and SIGVALD B REFSUM

Received 12 ii 65

Histological examination of jejunal mucosal biopsies is widely used for the aetiological differentiation between primary and secondary malabsorptive syndromes In idiopathic steatorrhoea the morphological changes may vary from almost normal mucosal pattern to the severe alterations with total villous atrophy A flat jejunal epithelium with an increased cellular infiltration extending to the muscularis mucosae has been considered most typical and specific for this primary malabsorptive syndrome (2, 3, 4, 6)

Recent reports, however, doubt the specificity of these changes (1, 5, 7) We have recently observed flat jejunal mucosal morphology in peroral biopsies from two patients with steatorrhoea secondary to sarcoidosis and lymphosarcoma, respectively The diagnoses in these cases were verified at autopsy

CASE REPORTS

Case 1 The patient was a 38 year old female who had parotitis in 1941 The following year diabetes mellitus was diagnosed and treated by insulin Retinopathy and nephropathy were observed in 1956 In November 1959 she was admitted to the Medical Department B Rikshospitalet Oslo because of diarrhoea which started in 1957 Increased amounts of fat and nitrogen were found in the stools on a fat balance diet 22 4 and 2 3 g per day respectively Neurological examination revealed neuropathy On treatment with glutenfree diet insulin vitamins and iron a remission occurred, *and the amounts of fat and nitrogen in the stools were normal after 5* weeks

Glutenfree diet was discontinued after three months because of its unpleasant taste After three years the patient was readmitted (March 1963) in a poor nutritional condition The insulin tolerance had been reduced during the last two months The laboratory tests revealed nephropathy with isostenuria proteinuria elevated serum kreatinin (2 2 mg/100 ml) and blood urea (138 mg/100 ml) and low serum proteins (5 4 g/100 ml)

Absorption studies showed increased faecal fat (56 8 g) and a nitrogen content of 6 7 g per 24 hours The daily weight of the stools was 800 g Malabsorption of fat was also revealed by the reduced plasma content of Vit A (605 I U per 100 ml Normal values above 800 I U) 4 hours after peroral administration of 350 000 I U of the palmitate A D xylose tolerance test was performed by giving 25 g of D xylose perorally She excreted 1 9 g in the urine during the following 5 hours normal values being above 4 5 g Reduced absorption of Vit B_1 was demonstrated by the

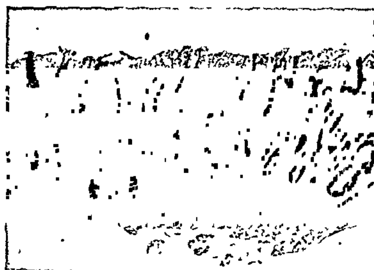


Fig 1
Jejunal biopsy section from case 1 Partly denuded mucosa with a flat appearence H + E staining × 50



Autopsy ... *Fig 2*

...ells in the

Schilling test In the 24 hour-urine 6 1 per cent of the test dose was found as compared to more than 10 per cent normally The secretin test after intravenous injection of 1 I U Secretin per kg bw showed normal figures for volume of secretion, output of bicarbonate and amylase

X-ray examination of the small intestine showed an abnormal pattern with dilated loops and flocculation of the barium suspension

A peroral jejunal biopsy obtained 70 cm from the teeth by means of a Crosby's

and that of the covering epithelium 21 microns as determined by a calibrated micrometer

Treatment with glutenfree diet was tried again but without improvement She

Case 2 The patient was a 71 years old female who had suffered from period ...

... She was very thin with oedemas and ...

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sto

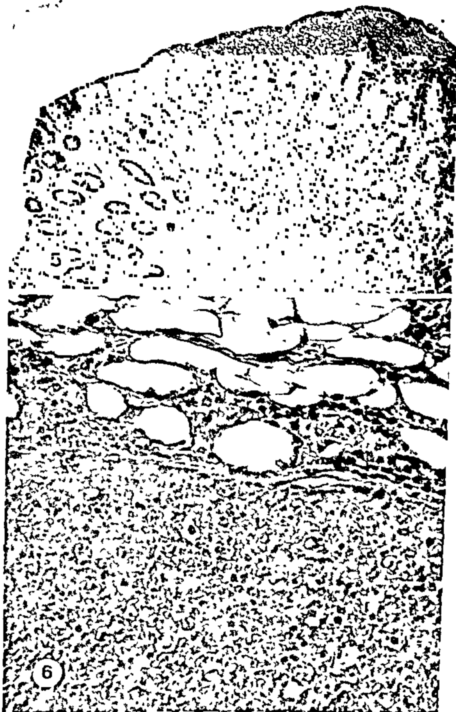
and ... as reduced, ... of plasma Severely impaired D xylose absorption was also found ...

... (Fig 5) There was a heavy infiltration of plasma cells and some lymphocytes and ...

Figs 3 4

Fig 3 Granulomatous lesions without necrosis in the liver H + E staining × 125 Case I

Fig 4 Trabecular fibrosis in a mesenterial lymph node H + E staining × 20 Case I



Figs 5 6

Fig 5 Jejunal biopsy section from Case 2 Partly denuded mucosa with a flat appearance H + E staining × 50

Fig 6 A lymph node with erradiated architecture and infiltration of lymphoblasts in the sinus and surrounding fat tissue × 320 Case 2

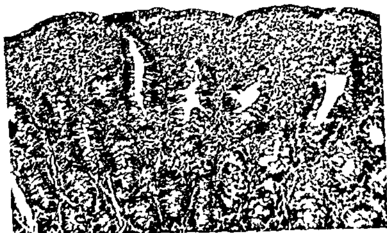


Fig 7
Autopsy jejunal mucosa with a heavy infiltration of lymphocytes and plasma cells in the lamina propria and a reduced height of the covering epithelium
H + F staining × 125 Case 2

DISCUSSION AND CONCLUSIONS

The persistent steatorrhoea and decreased absorption of Vit A, D xylose and labelled Vit B_{12} for more than 4 years prior to death in both of our patients indicated a primary malabsorption syndrome This suggestion was strongly supported by the findings of a flat mucosal epithelium in the jejunal biopsies, consistent with the diagnosis of idiopathic steatorrhoea, and by the improvement obtained with gluten free diet in one of the patients In both cases the cause of malabsorption and jejunal mucosal alteration was initially thought to be a combination of idiopathic steatorrhoea and diabetic enteropathy

The autopsy findings of changes consistent with Boeck's sarcoid in one case and malignant lymphoma in the other, however, may fully explain the malabsorption and the flat mucosal pattern demonstrated is

carcinoma in these cases severe infiltration of the submucosa with malignant cells was found (1) A flat mucosa is further reported in two children without coeliac disease (7)

It may thus be concluded that the finding of total villous atrophy in biopsy specimens from the jejunum is not specific for idiopathic steatorrhoea The mucosal lesions seen in our two cases may be secondary to the impairment of the lymph drainage and blood flow or due to local involvement of the primary disease However, the possibility also exists



Figs 5–6

Fig 5 Jejunal biopsy section from Case 2 Partly denuded mucosa with a flat appearance H + E staining × 50

Fig 6 A lymph node with erradiated architecture and infiltration of lymphoblasts in the sinus and surrounding fat tissue × 320 Case 2



Fig 7

Autopsy jejunal mucosa with a heavy infiltration of lymphocytes and plasma cells in the lamina propria and a reduced height of the covering epithelium H + E staining × 125 Case 2

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The autopsy findings of changes consistent with Boeck s sarcoid in one case and malignant lymphoma in the other however may fully explain the malabsorption and the flat mucosal pattern demonstrated is believed to be secondary to these diseases It seems to us very unlikely that they should also suffer from idiopathic steatorrhoea Similar mucosal atrophy has been reported in two patients after total gastrectomy for carcinoma In these cases severe infiltration of the submucosa with malignant cells was found (1) A flat mucosa is further reported in two children without coeliac disease (7)

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POST TRAUMATIC FAT EMBOLISM

Red Cell Aggregation, Hyaline Microthrombi and Platelet Aggregates in 5 Fatal Cases

By

ARNE SERCK-HANSSEN

Received 12 ii 65

The purpose of this communication is to draw attention to certain findings in the brains of 5 fatal cases of post traumatic fat embolism, giving support to some recent experimental and clinical observations on the pathogenesis of the microcirculatory disturbances in this condition

Post traumatic fat embolism is usually diagnosed clinically on the basis of characteristic symptoms occurring after trauma, usually fractures Although some pathologists, in order to make the diagnosis, require multiple small haemorrhages or haemorrhagic infarcts in the brain, the diagnosis is quite commonly made on the mere finding of intravascular fat globules

However, stainable intravascular fat globules can be found in a large variety of conditions unrelated to trauma (*Wagner* 1862, *Lehman* & *Moore* 1927) and the latter were of the opinion that fat globules might originate from plasma lipids This view is in opposition to the oldest and still prevailing concept of fat embolism, according to which fat of extravascular origin after trauma gains access to, and causes a blocking of the circulation in small vessels

Fat globules in the circulation have also been produced experimentally by methods not involving trauma to fat cells *Bergentz* (1961) injected Russel s viper venom and watery extract from rabbit brains and found changes in the blood lipids with formation of intravascular fat droplets *Adkins, Foster* & *O Saile* (1962) injected thrombin intravenously and found intravascular fat globules often associated with precipitates of fibrin and occlusive masses consisting largely of tightly packed, red blood cells

Hence the condition conventionally designated as post traumatic fat embolism cannot be diagnosed on the basis of intravascular fat globules alone, but must rest on the coexistence of multiple, small haemorrhages or haemorrhagic infarcts in the brain in association with a history of trauma

that they are secondary to the state of malabsorption and undernourishment that may be caused by the sarcoidosis and lymphosarcoma impairing the rapid turnover of cells in the small intestinal mucosa

SUMMARY

Two patients with diabetes mellitus and steatorrhoea of many years duration are described Peroral biopsy of the jejunal mucosa demonstrated a total villous atrophy in both patients At autopsy the diagnosis of sarcoidosis Boeck and lymphosarcoma, respectively were made It is concluded that the finding of total jejunal villous atrophy in biopsy specimens is not specific for idiopathic steatorrhoea

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The fat globules have generally been held responsible for the microcirculatory disturbances in post traumatic embolism However, *Bergentz* (1961) concluded from experimentally produced post traumatic fat embolism that the microcirculatory disturbances were not primarily caused by the fat emboli, but by the sludging effect on the red cells caused by alterations of the blood lipids *Gelin* (1956) had in earlier clinical and experimental work on anaemia after injury found that fractures, amongst other forms of trauma, resulted in intravascular aggregation of red cells, conditioning morphological renal and hepatic changes

It appears also that trauma to the blood itself may cause alterations in the serum lipids In 13 patients who all died after cardiopulmonary bypass, *Bleifeld* (1961) found intravascular sudanophilic material as well as silicon particles from the artificial tubes in the brains and kidneys, but only in 4 cases did he find focal changes in the brain These changes were morpohologically identical with those seen in post traumatic fat embolism The author regards the fat and silicon as the possible causes of the microcirculatory disturbances but comments on the large number of fat emboli in contrast to the few focal lesions

On the other hand, *Long, Sanchez, Varco & Lillehei* (1961) found that patients operated on under cardiopulmonary bypass exhibited a marked intravascular aggregation of red cells, and a tendency towards intravascular coagulation This has laso been found by *Penick, Averette, Peters & Brinkhous* (1958), who regards the haemorrhagic syndrome occasionally developing in these patients as secondary to intravascular coagulation which leads to reduction of thrombocytes and Factor VIII

Occasionally, in fatal cases following trauma, the classical multiple small haemorrhages in the brain can be demonstrated not withstanding the absence of intravascular fat (*Scutt* 1962)

It appears, therefore, that intravascular fat may be present without morphological changes in the brain and, perhaps more important, focal changes may be present without intravascular fat globules

These various reports further indicate that trauma, thromboplastic substances, or thrombin, may lead to the following changes in the blood

a) the formation of fat globules
b) a tendency towards red cell aggregation, and
c) intravascular coagulation

In practically all publications dealing with the problem of fat embolism, the attention has been focussed on the fat globules, both as regards the diagnosis and the genesis of the microcirculatory disturbances in this condition No morphological study on fatal cases of post traumatic fat embolism appears to have considered red cell aggregation and intravascular coagulation as possible causes of the microcirculatory

disturbance, although observations of both phenomena have been reported

In the present study an attempt has been made to find morphological changes, other than fat globules, which might explain the microcirculatory disturbances in fatal cases of post traumatic fat embolism

MATERIAL AND METHODS

The brains from 5 cases were studied All patients died following trauma combined with one or several fractures and multiple pin point haemorrhages in the brain were found in all cases at autopsy In Cases 1 2 and 3 nothing but paraffin blocks were available in Cases 1 and 2 from the cerebrum in Case 3 from the pituitary exclusively In Cases 4 and 5 the whole brain was available and frozen sections as well as paraffin sections have been examined Tissues from all cases were fixed in 10 per cent formalin Paraffin sections were stained with haematoxylin and eosin Lendrum's acid picro Mallory method for demonstration of fibrin (*Lendrum* 1949) and occasional sections with Weil's stain for the easy demonstration of ischaemic infarcts in the white matter In Cases 4 and 5 serial frozen sections were made from gelatin embedded tissue every 5th section was stained with Sudan *III for fat 120 sections* from each case being examined The frozen sections were made from brain tissue adjoining the areas from which paraffin sections were made

Case Reports

Clinical data and macroscopical findings in the brains are listed in Table 1

Microscopy of the brains All cases, except Case 3 *where the pituitary was the only material available*, revealed a large number of ischaemic and haemorrhagic infarcts, and appearant ball and ring haemorrhages These focal lesions were of varying age in one and the same case The lesions were most numerous in Case 1 (Fig 6), least numerous in Case 5, in which, however, anything up to ten lesions could be counted in the corpus callosum within a circular field with diameter 7 mm (low power field)

Apart from the focal lesions, the most striking finding in all cases was the great number of vessels, mostly venules and veins constaining densely packed, completely or partly hæmolysed red cells (Fig 7) This could also be seen in small arteries (Fig 8) Occasional, spherical, empty spaces with a diameter up to 30–40 μ were present between the erythrocytes, presumed to indicate dissolved fat globules This was not found in Case 5, although 120 sections were examined Occasionally the closely packed red cells appeared as fused, partly hyaline masses, so-called 'hyaline pseudothrombi' In addition to the packed red cells in the lumen of the vessels, a large number of the smaller venules and capillaries exhibited markedly swollen endothelial cells Around these vessels oedema, leucocytes and increased numbers of glial cells were observed (Fig 9) The combination of closely packed aggregates of red cells, reaction in the vessel wall and the perivascular tissue, is taken to imply stasis

TABLE 1

Tabulated Case Reports

Case No	1	2	3	4	5
Name sex age in years	O I ♂ 21	J R ♀ 80	S T ♂ 69	T O, ♂ 89	J P, ♂ 28
Autopsy no	1734/55	899/59	2023/60	400/62	862/64
Type of trauma	Fall from 20 meters	Traffic accident	Traffic accident	Traffic accident	Fall from 5th floor on to street
Injuries sustained	Fractured skull both femoral shafts patella one meta carpal bone and lower jaw	Fractured r humerus r tibia and fibula and pelvis	Fractured pelvis r ulna r tibia and fibula Rupture of mesentery of small intest and contusion of the liver	Fractured r tibia and fibula	Fractured pelvis, 5 ribs radius and ulna Rupture of liver and bladder Contusion of the lung
Shock	On admission	1 hr after admission	On admission	5 days after admission	On admission
Petecchiae	On 2nd day	On 2nd day	Not commented on	On 5th day	None
Survival period	6½ days	7 days	3 days	6 days	2 days
Macroscopic lesions in the brain	Unusually large number of hemorrhagic points in the white matter (Fig 1)	*Large number of* haemorrhagic points in the white matter (Fig 2)	*Large number of* haemorrhagic points in the white matter (Fig 3)	Numerous haemorrhagic points in the white matter (Fig 4)	Scattered haemorrhagic points in the white matter (Fig 5)

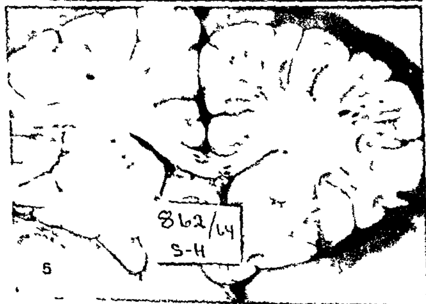


Figs 1 2

Fig 1 Cr sect n f cerebrum Case 1 Unusual large number of f cal les ons
Fig 2 Cr sect n of cerebrum Case 2 Large number of focal lesions

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Figs 4 5

Fig 4 Cross section of cerebrum Case 4 Numerous focal lesions
Fig 5 Cross section of cerebrum Case 5 Scattered focal lesions

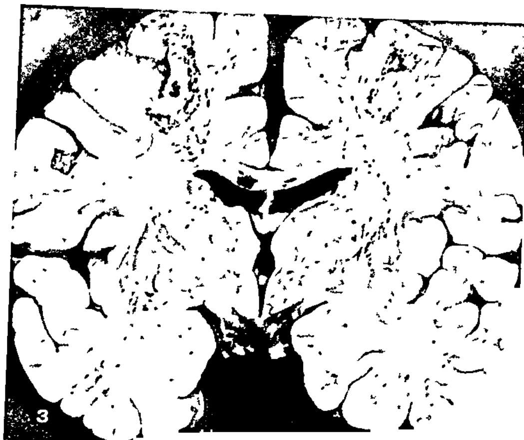


Fig 3
Cross section of cerebrum Case 3 Large number of focal lesions

All cases except No 1, revealed a large number of venules and arterioles containing small hyaline bodies which stained brilliantly red with Lendum's stain These bodies were perfectly round with a distinct, highly refractile surface Their size varied from a few up to 30–40 μ Although, the bodies were usually homogenous, scattered small vacuoles could occasionally be seen, usually near the centre (Fig 10) In frozen sections the hyaline bodies had a surface which was slightly more sudanophilic than the centre that stained light yellow, apart from occasional central sudanophilic spots The latter had the same size as the vacuoles found in paraffin sections In addition a quite narrow band composed of small sudanophilic spots formed a concentric ring parallel to the surface of the body, about ⅓ of the way from the periphery to its centre (Fig 11) In Case 3, from which the *pituitary was the only block* available, some of these bodies had fine threads radiating from their surface The threads also stained red with Lendrum's stain (Fig 12) Hyaline bodies were found partly alone, partly in groups, sometimes amongst what appeared to be hæmolysed red blood cells (Fig 13), and occasionally in close proximity to aggregates of platelets (Fig 14)

All cases except No 3, revealed occasional small vessels, usually venules, containing loose aggregates of platelets They were found either alone, or in close association with Lendrum-positive bodies, but rarely associated with fibrin precipitates of usual morphology They were usually floating free in the lumen, and rarely adherent to the wall

According to the autopsy report frozen sections from Cases 1, 2 and 3 revealed intravascular fat globules In Case 4, frozen sections stained with Sudan III, revealed a fair amount of fat in the cerebral vessels, but there appeared to be no more fat in the vessels in the region of the focal lesions than there was in other areas, and usually no reaction was *evident in the vessel wall where the fat was found* In Case 5, 120 frozen sections stained with Sudan III were examined, 60 of these were from the corpus callosum No intravascular fat globules were found

DISCUSSION

The 5 cases cited above present a fairly uniform clinical and pathological pattern They all sustained injuries, mostly severe, accompanied by one or several fractures One patient (Case 1) has a fractured skull, but evidence of direct trauma to the brain was not seen in any of the cases Although to different degrees, all cases presented multiple small haemorrhagic points in the white matter of the brain

The following microscopic observations were made in the brains

1) Multiple, diffusely scattered haemorrhagic and occasional, ischæmic infarcts, ring and ball haemorrhages (all cases, except No 3, *where the pituitary was the only block available*
2) Widespread red cell aggregation in the capillaries, arterioles and venules (all cases)
3) Intravascular, Lendrum-positive bodies (Case 2, 3, 4, 5)
4) Aggregates of platelets (Case 1, 2, 4, 5)
5) Intravascular lipid material (all cases, except No 5)

The Infarcts and Haemorrhages

The mixture of ischæmic and haemorrhagic infarcts are interesting If only intraluminal causes of occlusion are considered, the infarcts may be explained in the following ways

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Figs 6 9

Fig 6 S...

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s stain

Fig 8 Densely packed haemolysed and partly necrotic red cells in small artery Case 4 Lendrum s stain × 230

Fig 9 Venule with densely packed haemolysed red cells and swallen endothelial cells Perivascular oedema leucocytes and glial cells Small hyaline micro thrombus arrow Case 5 Lendrum s stain × 230


6
9

The ischæmic infarcts may be regarded as the result of a permanent occlusion on the arterial side, together with a venous drainage from the surrounding area which is sufficient to prevent any venous backflow The ischæmic infarcts are rare in comparison with the haemorrhagic infarcts

The haemorrhagic infarcts may have been produced either by

1) temporary occlusion on the arterial side leading to necrosis of the brain parenchyma ond damage to the vessel wall, followed by re establishment of the local circulation, or by
2) markedly hampered venous return from areas which are injured by a permanent arterial occlusion

The clinical cerebral symptoms are commonly fluctuating This suggests an intermittent circulatory disturbance which may be explained by the first hypothesis

The haemorrhages take form either of ring haemorrhages or ball haemorrhages when a single section is examined However, by serial section we have shown that the ball haemorrhages usually represent a peripheral section through a ring haemorrhage, *i e* there is usually no true difference between the two types A ring haemorrhage usually has an area of necrosis in is centre, and is probably nothing but an haemorrhagic infarct

It is possible, however, that some ball haemorrhages *may be genuine* haemorrhages without infarcts, secondary to anoxic damage to the vessel wall *An other* possibility is that they may be secondary to a haemorrhagic diathesis induced by *the trauma Gelin (1956)* found a significant decrease in thrombocytes during the first five days after various kinds of trauma, and *Penick, Avrette, Peters & Brinkhaus* (1958) relate the haemorrhagic syndrome following the use of extracorporeal circulation to *the consumption of* platelets and Factor VIII The initiation of this *process depends* on the presence of active thromboplastic substances in the blood stream

As in previous reports, most haemorrhagic lesions in the brain were

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Figs 10-14

Fig 10 Two hyaline microthrombi with several small vacuoles Case 2 Lendrum's stain × 550

Fig 11 Hyaline microthrombi of varying size in small artery Concentric sudanophilic band in large thrombi Central sudanoph[illegible] arrows Cas[illegible]

Fig 12 [illegible]

Fig 13 [illegible]

Fig 14 [illegible]


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the underlying cause has been obscure He regards them as the primordial body in many cases of intravascular coagulation and he has proposed the name hyaline microthrombi The finding of aggregates of platelets in close association with some of these hyaline microthrombi makes it likely that the latter are formed during life (Fig 14) The radiating fibres with staining properties of fibrin which arise from their surface (Fig 12) lend additional support to the assumption that they may represent fibrin precipitates although of unusual morphology The frozen Sudan stained sections revealed occasional sudanophilic spots in these bodies which in distribution and size were similar to the vacuoles observed in paraffin sections (Fig 10 and 11) These vacuoles may therefore be spaces after degenerated platelets which are rich in lipids

The role of the hyaline microthrombi in the genesis of the microcirculatory disturbances in post traumatic fat embolism is uncertain their occurrence is mainly taken to indicate that intravascular coagulation and possible fibrinolysis or defective polymerization of fibrin is taking place (*Alkjaersig Fletcher & Sherry* 1962 *Bang Fletcher Alkjaersig & Sherry* 1962 *Skjorten* 1964) Fibrin thrombi of usual morphology have also been seen in cases of post traumatic fat embolism In a fatal case a young man *Russel* (1941) found a large number of capillaries apparently blocked by fibrin thrombi as well as thrombotic occlusion of an meningeal artery *Waaler* (1943) noted the presence of fibrin thrombi adjacent to lipid material in the glomerulus of the kidney and *Cammermeyer* (1953) noted fibrin adjacent to lipid material intravascularly in the brain and the glomeruli The last two workers are inclined to regard the fibrin precipitation as a secondary manifestation either to the blocking of the vessels by lipids or to the release of thromboplastic substances from multiple necrotic foci notable in the brain

Aggregates of Platelets

Aggregates of platelets were found either alone in the vessels or in close association with the hyaline microthrombi The aggregates were mostly seen in the venules This suggests that they are formed locally They were rarely seen to adhere to the wall *Jorgensen* (1964) found evidence of local formation of platelet aggregates in flowing blood independent of the wall and possibly secondary to intravascular coagulation

However an embolic genesis can not be excluded when the aggregates are found on the arterial side *Bergent* (1961) noted in *in vivo* studies white emboli considered to contain platelets in vessels at considerable distance from traumatized areas and he noted that these emboli could cause partial occlusion and decreased flow A possible explanation is that the aggregates of platelets may be formed secondary

found in the white matter A discussion of their distribution is considered to be outside the scope of the present paper

Red Cell Aggregation (Stasis)

The diagnosis of stasis in histological sections is not easy, but the finding of vessels containing tightly packed hæmolysed red blood cells, in contrast to neighbouring vessels with red cells of normal appearance, is very suggestive When also a reaction from the vessel wall, evident by a marked swelling of the endothelial cells, and perivascular oedema is present, the changes must be premortal (Fig 9) The finding of hyaline "pseudothrombi" which must be regarded as closely packed, necrotic red cells, is taken as definite evidence of stasis

The observation of stasis causing microcirculatory disturbance is old, and among others described by *Lister* in 1885 Most publications on stasis deal with a local condition secondary to injury to the vessel wall, as seen in inflammatory reaction following a noxious stimulus The close association between local stasis and necrosis has been stressed by *Kreyberg* in several publications (1963, review)

Less generally accepted is the hypothesis of stasis as a general phenomenon occurring in vessels remote from injured areas In his monograph on fat embolism *Grondahl* (1911) described capillaries and small arteries containing homogenous small hyaline thrombi that completely filled their lumen and occasionally contained fine radiating threads of fibrin He did not discuss their possible causative rôle in the circulatory disturbance From the description it appears that the hyaline thrombi probably represented the hyaline "pseudothrombi" seen in stasis

Knisely, Eliot & Bloch (1945) introduced the term "sludge" for the phenomenon of red cell aggregation, and postulated a "sludge initiator substance"

Gelin (1956) found that fractures, burns and contusions were followed by intravascular aggregation of red cells that resulted in morphological changes in tissues far from the local injury He also found that infusions of high molecular dextran caused red cell aggregation and impaired capillary flow It is therefore unlikely that the microcirculatory disturbances observed at some distance from the site of trauma was necessarily dependent on a nervous mechanism

In the case of post traumatic fat embolism the release of thrombo plastic substances from the traumatized tissues induces changes in the serum lipids with the formation of fat droplets These fat droplets may well act as "sludge initiator substance'

Intravascular Lendrum Positive Bodies

Intravascular Lendrum positive bodies have been found and described in detail by *Skjorten* (1964) in cases of bilateral renal cortical necrosis, in septic and circulatory shock and also in conditions where

the underlying cause has been obscure. He regards them as "the primordial body in many cases of intravascular coagulation", and he has proposed the name hyaline microthrombi. The finding of aggregates of platelets in close association with some of these hyaline microthrombi makes it likely that the latter are formed during life (Fig 14). The radiating fibres with staining properties of fibrin which arise from their surface (Fig 12) lend additional support to the assumption that they may represent fibrin precipitates, although of unusual morphology. The frozen, Sudan-stained sections revealed occasional sudanophilic spots in these bodies which in distribution and size were similar to the vacuoles observed in paraffin sections (Fig 10 and 11). These vacuoles may, therefore, be spaces after degenerated platelets which are rich in lipids.

The rôle of the hyaline microthrombi in the genesis of the microcirculatory disturbances in post traumatic fat embolism is uncertain, their occurrence is mainly taken to indicate that intravascular coagulation and possible fibrinolysis or defective polymerization of fibrin is taking place (*Alkjaersig, Fletcher & Sherry* 1962, *Bang, Fletcher, Alkjaersig & Sherry* 1962, *Skjorten* 1964). Fibrin thrombi of usual morphology have also been seen in cases of post traumatic fat embolism. In a fatal case, a young man, *Russel* (1941) found a large number of capillaries apparently blocked by fibrin thrombi as well as thrombotic occlusion of an meningeal artery. *Waaler* (1943) noted the presence of fibrin thrombi adjacent to lipid material in the glomerulus of the kidney, and *Cammermeyer* (1953) noted fibrin adjacent to lipid material intravascularly in the brain and the glomeruli. The last two workers are inclined to regard the fibrin precipitation as a secondary manifestation, either to the blocking of the vessels by lipids, or to the release of thromboplastic substances from multiple necrotic foci, notable in the brain.

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to the formation of thrombin following trauma In the present cases platelet aggregates were not an infrequent finding, but less conspicuous than both stasis and hyaline microthrombi Their presence is taken as a further indication that intravascular coagulation is taking place

Intravascular Lipid Material

Intravascular lipid material was found in all cases, *except Case No 5* The lack of intravascular fat in the last case may be an important observation Even if the number of infarcts in the brain in this case was fewer than in the other cases, it does show that intravascular fat globules are not necessarily present, and, therefore, probably not required for the development of the typical focal lesions This lends support to the view expressed by *Bergentz* (1961) that the clinical symptoms ascribed to fat embolism, and consequently the microcirculatory disturbances, are not caused by the fat globules On the whole, we feel that no proof has been published that intravenous injection of small amounts of autologous fat does cause a blocking of the vessels *Lehmann & Moore* (1927) found that intravenous injection of all the fat from a femur, not necessarily was sufficient to kill the experimental animal A further indication that the fat globules are not occlusive, is the absence of reaction in the vessel wall where the fat globules are found, an observation commented on by many workers

An interpretation of the relative importance of the various factors cited above in the genesis of the microcirculatory disturbances after trauma is difficult, but red cell aggregation was the most frequent and dominating finding in the cases presented here

Intravascular coagulation with fibrin precipitation and platelet aggregation were quantitatively less impressive, but their relative importance may well vary from case to case

On the whole, we feel that the observations cited above lend support to the view that fat is not the only factor to be considered as the cause of the microcirculatory disturbance after trauma, red cell aggregation and intravascular coagulation may well be more important

SUMMARY

The brains of five fatal cases of post traumatic fat embolism have been examined with a view to find factors other than intravascular fat which might provide en explanation of the microcirculatory disturbances

Red cell aggregation and intravascular coagulation in the form of hyaline microthrombi and aggregates of platelets were observed, and are considered possible causes of the microcirculatory disturbances Red cell aggregation with stasis is considered the most important, single factor

admitted for minor surgery No one known to have hepatitis or any blood disease was included

Preparation of Lymphocytes

The sterile technique devised by *Brent & Medawar* (1963) was used In all cases the lymphocytes were counted immediately before the injection As a rule the sus [illegible] and about 5 million lympho [illegible] cent of the lympho [illegible]

Skin Window Technique

[illegible]

covered with the first of a series of sterile cover slips changed every [illegible] hours through at least 48 hours The cover slips are stained by the method of May-Grunwald Giemsa mounted in Canada balsam on slides and examined microscopically At least 200 cells were counted More than 1200 preparations were examined

Injection of Increased Quantities of Lymphocytes

Fifteen subjects were injected with lymphocytes in amounts ranging from 195 000 to 16 *millions* As the reaction to the same lymphocytes varies widely from one person to another four skin window experiments were usually performed on the same recipient injecting lymphocytes of one and the *same sample The quantity* was varied by diluting the sample with physiological saline In experiments requiring more than 5 million lymphocytes up to 0 3 ml of lymphocyte suspension was injected In *most* cases an intracutaneous test with lymphocytes and macroscopic reading of the reaction at the end of 48 hours was carried out simultaneously with the skin window experiments The *reaction* was assessed microscopically on the basis of the average percentage of basophilic leucocytes in five preparations showing the strongest reaction

Injection of Disrupted Lymphocytes

Into seven persons *lymphocytes* which had been subjected to freezing and thawing six times were injected On trypan blue staining a maximum of 15 per cent of the lymphocytes were found to be viable In experiments on three persons injected with lymphocytes treated with *sterile water* less than 35 per cent of the lymphocytes took up trypan blue At the same *time control* experiments were carried out on the same patients using untreated lymphocytes of the *same sample*

Injection of X Ray Irradiated Lymphocytes

X radiated lymphocytes were injected into 13 persons immediately *after the* irradiation into *4 persons 72* hours after the irradiation the lymphocytes being stored in the meantime at 37° C *in a culture* medium without addition of phyto haemagglutinin Immediately after the X radiation more than 95 per cent of the lymphocytes were viable as demonstrated by trypan blue staining but at the end of 72 hours less than 10 per cent were viable The radiation factors were as *follows* 140 kV 20 mA 4 mm Al *filter 30 cm* distance dosimeter and a screen behind the lymphocytes when the dosage was close to 5000 r At a higher dosage the factors were *100 kV* 20 mA 0 filter 13 cm irradiation distance without using a tube Tube capacity calculated as 1700 r min when irradiating with a screen behind the lymphocytes The lymphocytes received *a radiation* dosage of from 152 to 51,000 r

RESULTS

The positive macroscopic reactions were characterized by induration and erythema It was often difficult to evaluate and assess the reaction

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DEPENDENCE OF THE NLT-TEST UPON QUANTITATIVE AND QUALITATIVE CHANGES OF THE INJECTED LYMPHOCYTES

By

PER WOLF-JURGENSEN

Received 22 ii 65

The *NLT* test (the normal lymphocyte transfer test) was described in 1963 by *Brent & Medawar* as a simple method by which it might be predicted which one among a number of donors is best suited to give a homograft to a recipient Intracutaneous injection of homologous lymphocytes from the perepheral blood is followed, in 24–48 hours, by a gross reaction characterized by induration and erythema This reaction is presumably a graft-*versus*-host reaction caused by the injected, small lymphocytes which are immunologically competent

Using this method on human subjects *Gray & Russel* (1963) could confirm *Brent & Medawar*'s results *Bridges, Nelson & McGeown* (1964), using lymphocytes from uraemic patients, found that lymphocytes from 3 of 7 patients produced no reaction *Wolf-Jurgensen* (1962) demonstrated that a typical delayed reaction, experimental allergic contact dermatitis, was characterized by basophilic leucocytes in the exudate *Wolf Jurgensen & Schwartz* (1964) also observed, in experiments using the skin-window technique, that basophilic leucocytes were a constant finding in the NLT test and a valuable supplement to the gross findings which may be difficult to assess because of a non-specific reaction

In the present paper the author reports the results obtained in a study in which the skin window technique was used to investigate the dependence of the NLT reaction upon quantitative and qualitative changes in the injected lymphocytes In some experiments the quantity of the injected lymphocytes was varied and in others the lymphocytes used were disrupted after repeated freezing and thawing addition of sterile water, or X-radiation In each experiment the reaction was assessed on the basis of the quantity of basophils in the preparations

MATERIAL AND METHODS

The material comprises a total of 42 persons 29 males ranging in age from 16 to 74 years and 13 females aged 21 to 54 years Most of the subjects were patients

Supported by a grant from the *Danish Science Foundation*

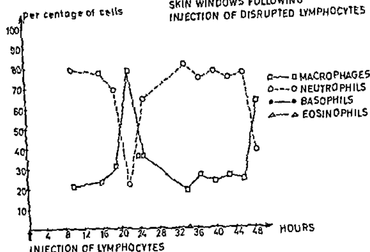


Fig 2

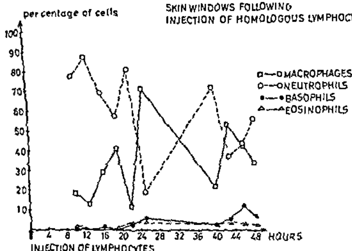


Fig 3

Figs 2 and 3

Cellular sequence in the exudate after injection of 5×10^6 disrupted and normal lymphocytes in a characteristic experiment on one recipient Basophilic leucocytes were not found in the *exudate* upon injection of disrupted lymphocytes, only in the experiment in which 5×10^6 *untreated lymphocytes* from the same donor were injected

treated lymphocytes from the same sample resulted in the finding of a maximum of 2 to 12 *per cent* basophilic leucocytes Figs 2 and 3 illustrate a characteristic experiment

In all the experiments using injection of lymphocytes, which received a radiation dosage *of* from 152 to 51 000 r, (Table 2) immediately after

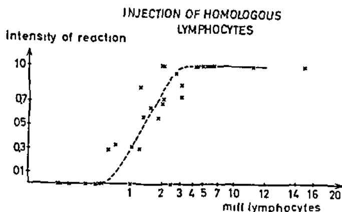


Fig 1

Dependence of the NLT reaction upon the quantity of injected lymphocytes Each dot represents an experiment The reaction was assessed on the basis of the percentage of basophilic leucocytes in the exudate

quantitatively The diameter of the induration was found to range from 2 to 8 mm The most pronounced reactions also showed the largest number of basophilic leucocytes in the exudate

The result of experiments using increasing amounts of lymphocytes is depicted in Fig 1 Each dot represents an experiment Injection of *more than 4 million lymphocytes does not lead to a more pronounced* reaction If less than 4 million lymphocytes are injected, the reaction decreases, and if less than ½ million is injected there will be no basophils in the skin-windows

TABLE 1

Tabulation of Experiment in which Disrupted Lymphocytes were Injected

Injection of disrupted homologous lymphocytes						
Manner of disruption	Disrupted lymphocytes			Control normal lymphocytes		
	Number	Basophilia	Negative	Number	Basophilia	Negative
Treatment with sterile water	3	0	3	3	3	0
Freezing and thawing	7	1	6	7	7	0

The reaction is designated as negative if the basophilic leucocytes in the exudate accounted for 1 5 per cent or less

Experiments using injection of lymphocytes disrupted by repeated freezing and thawing (Table 1) failed to elicit any reaction in six cases (less than 1 per cent basophilic leucocytes in the exudate) but gave a weak reaction (2 5 per cent basophils in one case) The same lympho cytes, if untreated gave rise to reactions between a maximum of 3 5 and 9 per cent basophilic leucocytes in the exudate

Experiments using injection of lymphocytes disrupted by the addition of sterile water induced no reaction (less than 0 5 per cent basophilic leucocytes in the skin windows), while experiments using un-

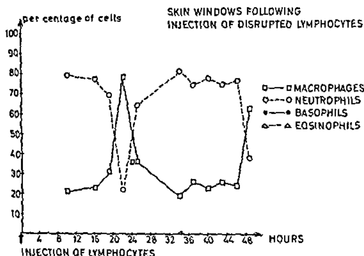


Fig 2

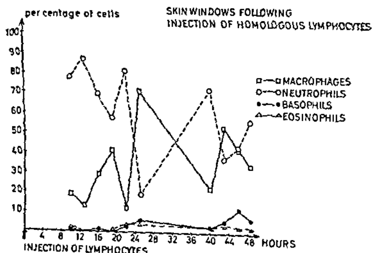


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In all the experiments using injection of lymphocytes, which received a radiation dosage of from 152 to 51 000 r, (Table 2) immediately after

the irradiation the reaction was exactly the same as the one seen when un-irradiated lymphocytes were used (from a maximum of 2 to 12 per cent basophilic leucocytes in the exudate). On the other hand, if the lymphocytes had been stored for 72 hours after irradiation with 4,500 r, there was hardly any reaction in the skin (less than 1 per cent basophils in the exudate), while the same lymphocytes untreated and stored for 72 hours elicited a reaction of from 1.9 to 9 per cent basophils.

TABLE 2

Tabulation of the Experiment in which X radiated Lymphocytes were Injected

Injection of homologous X irradiated lymphocytes						
Time after X irradiation	X irradiated lymphocytes			Control normal lymphocytes		
	Number	Basophilia	Negative	Number	Basophilia	Negative
Immediately	36	36	0	13	13	0
72 hours	4	0	4	4	3	1

The reaction is designated as negative if the basophilic leucocytes in the exudate accounted for 1.5 per cent or less.

DISCUSSION

Basophilic leucocytes in the exudate have previously been demonstrated as a constant finding in experimental, allergic contact dermatitis induced by 2,4 dinitrochlorbenzene (*Wolf Jurgensen* 1962, *Aspegren et al* 1963). The present experiments confirmed previous results (*Wolf-Jurgensen & Schwartz* 1964), *viz* that *injection of homologous lymphocytes into the skin induces an allergic reaction of a delayed type characterized by basophils in the exudate*. Morphologically, the cells are similar to basophilic leucocytes in the blood. Immature forms are not seen. Thus, the antigen antibody reaction cause an increased number of basophils migrate from the vessels at the site of the reaction and/or bind those basophils which pass from the blood at the site of reaction. In non-specific inflammations they are not present (*Rus* 1959). The reaction is due to injected immunologically competent cells, the small lymphocytes (*Medawar* 1963). The transferred lymphocytes must be assumed to form antibodies to foreign tissue antigens. That antigen-antibody reaction is not caused by autologous lymphocytes accords with the theory of clonal selection (*Burnet* 1959).

The system seems to be saturated when 4 million lymphocytes or more are injected. Using an agar plate technique *Jerne et al* (1963) demonstrated that the number of antibody-forming cells in the spleen will increase to about although not above 100,000 after i.v. injection of 10^{7} sheep erythrocytes into mice, even though the quantity of injected sheep blood cells is increased. Also in this case, the system seemed to be saturated, even specifically, as injection of another antigen increased the antibody forming cells in the spleen.

The present experiments show also that the reaction is caused by living lymphocytes as non vital lymphocytes remnants of non vital lymphocytes or the suspension medium fail to elicit any reaction It was demonstrated by *Landsteiner & Chase* in 1942 that cytoallergy may be transferred experimentally with sensitized vital lymphocytes

Lawrence (1961) found that delayed hypersensitivity may be transferred with cell free solutions after freezing and thawing of the sensitized lymphocytes or addition of hypotonic fluid Lawrence suggested that it might be a question of a "transfer factor", a reactive locus in the lymphocytes which in the presence of hypersensitivity by an antigen has been stimulated to the formation of an increased number of lymphocytes with a reactive locus directed against the specific antigen Upon entering into the organism the antigen (X) is *imagined to be* phagocytized by macrophages and with the *macrophage* (self) to form a complex (self X) which stimulates the reactive locus in the lymphocytes *The* results of the present investigations showing that the delayed reaction cannot be performed with cell free suspensions of the lymphocytes is not at variance with this finding as there is no question of transferring hypersensitivity Normal transplanted lymphocytes have not previously been stimulated by antigens from foreign tissues

X radiation has no immediate effect upon the lymphocytes which are capable of inducing a reaction just like un irradiated lymphocytes But 2 hours later they induce practically no reaction The mean 50 per cent survival time following irradiation with 1000 r has been found to be 1 7 days (*Schrek et al* 1962) Un irradiated lymphocytes which are merely stored for 72 hours elicit a reaction *Viable lymphocytes is a* prerequisite for the reaction Probably there is a preformed capacity to manufacture antibody which will exist in the damaged cell *at least for* a brief period Investigations by the skin window technique may be a valuable method by which to assess *quantitatively* antigen antibody reactions of the delayed type

SUMMARY

The dependence of the NLT *test upon the* quantity of injected lymphocytes and experiments in which non vital lymphocytes were injected were performed by *the skin* window technique The reaction was assessed microscopically *on the* [illegible]
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THE DISTRIBUTION OF INTESTINAL METAPLASIA IN MACROSCOPIC SPECIMEN, DEMONSTRATED BY A HISTOCHEMICAL METHOD

By

R I Graham and R O K Schade

Received 25 ii 64

The intestinal metaplasia or "Becherzellenmetaplasie" of the German literature as observed in the gastric mucosa has been known for a long time It has been described in detail as an interesting histological phenomenon but up to this date the true significance of this lesion is uncertain We have, for example, but little knowledge of its effect on the digestive processes We know of the common association of intestinal metaplasia with chr atrophic gastritis and achlorhydria when the morphological changes are widespread

As far as one can see the first mention of intestinal metaplasia is to be found in a paper by *Kupffer* in 1883 The lesion was then considered to be a normal constituent of the gastric mucosa *Boas* found fragments of mucosa with intestinal metaplasia in specimens of gastric lavage The main argument centring around this condition consisted in deciding whether one was dealing with a congenital heteropia of intestinal epithelium or with a true metaplasia of gastric epithelium acquired during life The latter view is accepted by the majority of investigators According to *Magnus* "the presence of intestinal epithelium in the stomach is the result of faulty regeneration of surface epithelium in a mucosa repeatedly damaged by gastritis and is an example of metaplasia resulting from chr irritation"

The possible relationship of this lesion to peptic ulceration and neoplasia has been neglected for a considerable length of time

Schmidt in 1896 described a mucoid carcinoma with goblet cells and cells with a striated border In addition, he noted that the mucus of the goblet cells stained in the same fashion as intestinal mucus In 1912 *Gosset & Masson* described adenomatous polyps of the pyloric antrum, made up of an intestinal type of epithelium In 1951 *Jarvi & Laurén* published a study on the relationship of intestinal metaplasia and car-

We wish to acknowledge with thanks the aid given to this investigation by a grant from the *North of England Council of the British Empire Cancer Campaign*
Paper given at the gastro intestinal seminarium at Uraj̈arvi (Finland) 28 8 1964

nants, and the suspension medium were unable to induce a delayed allergic reaction upon intracutaneous injection

Basophilic leucocytes in the exudate are a constant finding in delayed allergic reactions and investigation by the skin window technique appears to be a valuable method by which to assess the NLT reaction quantitatively This method might well prove useful in further studies on the immunologically competent cells and antigen-antibody reactions of the delayed type

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shows on histological examination a strong alkaline phosphatase reaction, we applied this method which we found most satisfactory. It offers two advantages. 1 formalin fixed material can be used, provided the reaction for the presence of the alkaline phosphatase is performed after not more than 24 hours of fixation and after adequate washing in running water 2 the materials necessary for the substrate are cheap and allow, therefore, the use of large quantities The technique, a modified coupling azo dye method after *Pearse*, runs as follows:

1 24 hour fixation (The specimen is opened along the larger curvature and pinned onto a clean cork board and immersed into fresh neutral formol saline)
2 Washing 2 3 hours in running water The specimen is removed from the cork board and allowed to float in the running water)
3 Immersion of whole specimen into substrate 0 1 Molar "Tris' buffer (pH10) containing 1 mg/ml sodium naphthyl phosphate and 1 mg/ml fast Red TR (5-chloro o toluidine)— (*George Gurr*)
4 Incubation at room temperature

It is not surprising that the reaction for alkaline phosphatase produces a striking picture in the macroscopic gastric specimen when one studies a microscopic section of intestinal metaplasia stained for alkaline phosphatase activity (Fig 1) The reaction takes place mainly within the free striated border of the cells

CASE REPORTS

Six cases have been chosen to illustrate the distribution and extent of intestinal metaplasia as demonstrated by the above described method

Case 1 The patient a woman of

Case 2 The patient a man of 69
the last 3 years Barn
f this a Billroth I p
The operative speci
curvature The intest
large parts of fundal

Case 3 The patient a woman of
appetite for 1 year and general
diagnosis of carcinoma of the stor

cinoma and concluded that "gastric tumours in the majority of cases originate in the heterotopic islands of intestinal epithelium" *Morson* in *1955 defined intestinal metaplasia* as follows 1 goblet cells must be present These columnar cells must contain mucus which stains blue with Ehrlich's haematoxylin and red with Southgate's mucicarmine 2 The lining epithelium of the tubules should bear a striated or brush border 3 Paneth cells and argentaffin cells should be identifiable in small numbers

With the development of histochemical techniques it appeared feasible to define further the nature of *intestinal metaplasia* and its possible bearing on neoplasia and ulcerogenesis

Wattenberg, Minneapolis, described in 1959 that the epithelium of *intestinal metaplasia gave an intensely positive reaction for aminopeptidase*, a reaction also strongly positive in the epithelium of the intestine The azo dye was largely localised at the luminal borders of the cells *He also described that in 7 out of 20 gastric carcinomata there was* marked aminopeptidase activity This paper was followed by a publication by *Planteydt & Willighagen* on the enzyme histochemistry of the human stomach *with special reference to intestinal metaplasia, using* a battery of enzyme reactions They demonstrated that the alkaline and acid phosphatase, the aminopeptidase, the glucose-6-phosphate dehydro genase and other enzyme reactions showed an identical response *in* duodenal epithelium and the areas of intestinal metaplasia, thereby proving that these two epithelia are not only morphologically but also histochemically identical

A further publication by *Morson* must be mentioned which largely stimulated our attempts of the study of intestinal metaplasia in the macroscopic specimen

Morson used the "swiss roll" technique, that means long, rolled up strips of gastric wall including mucosa or gastric mucosa only,—a technique first used by *Magnus* in *1937*—to study the distribution of the intestinal metaplasia and to arrive at a quantitative assessment of it He concluded—and I quote *Morson*—that cancerous stomachs contain more than four times the amount of intestinal metaplasia *than the* stomach specimen removed for duodenal ulcer and nearly twice the amount seen in cases of gastric ulcer even when allowing for the fact that metaplasia appears to increase with age

These attempts of quantitation of intestinal metaplasia and the advent of histochemistry persuaded us to try histochemical methods on the whole fresh operative specimen *By such a procedure we hoped to* facilitate an estimation of the areas involved by metaplasia and to find its topographic distribution as well as its relation to ulcer and carcinoma alike

We attempted first of all the demonstration of the leucine aminopeptidase For a variety of technical reasons this method proved itself unsatisfactory and in *addition very costly As the intestinal metaplasia*

Fig 5





Fig 6



Fig 7

Case 4 The pat ent a man of 56 complained of upper abdom nal pain for 3 years A Barium meal showe l a large healing lesser curve ulcer A Billroth I partial gastrectomy was performed I neventful recovery

The specimen (Fig 3) showed almost at the line of excision a chr peptic ulcer of typical appearance with muccsal folds radiating towards it The entire antrum and part of the fundal mucosa was the seat of intestinal metaplasia and showed the characteristic br wn c l ur of a positive reaction Again the ulcer was situated



a filling defect confirmed the clinical impression A laparotomy was performed At operation a tumour could not be detected but a lesser curve ulcer was identified and a Polya gastrectomy performed

The operative specimen (Fig 2) showed an antral intestinal metaplasia mainly confined to the pyloric canal reaching to and surrounding a chr peptic ulcer

Fig 5





Fig 6



Fig 7

Case 4 The patient a man of 56, complained of upper abdominal pain for 3 years A Barium meal showed a large healing lesser curve ulcer A Billroth I partial gastrectomy was performed Uneventful recovery

The specimen (Fig 3) showed almost at the line of excision a chr peptic ulcer of typical appearance with mucosal folds radiating towards it The entire antrum and part of the fundal mucosa was the seat of intestinal metaplasia and showed the characteristic brown colour of a positive reaction Again the ulcer was situated

within the area of intestinal metaplasia and therefore within an area of chr gastritis

Case 5 The patient a man of 77, had been under treatment for pernicious anaemia since 1948 The pernicious anaemia was well controlled However, in July 1962 the patient was admitted with a secondary anaemia which did not respond to treatment There was no loss of weight No gastric symptoms Routine gastric cytology was positive for malignant cells and therefore, indicative of carcinoma Subsequent radiological examination showed a filling defect in the upper part of the greater curvature A high Polya gastrectomy was performed The patient died a few days after the operation

The specimen (Fig 7), opened along the lesser curvature, showed a large sessile carcinoma within the fundal region and in the greater curvature In pernicious anaemia the main mucosal changes—including intestinal metaplasia—are seen in the fundal mucosa This is borne out by this specimen where almost the entire fundal mucosa is the seat of intestinal metaplasia while the antrum is almost free of any change

Case 6 The patient, a man of 59, was admitted as an emergency complaining of pain in the r and l hypochondrium The presence of a gastric carcinoma was demonstrated by radiology A laparotomy followed by a partial gastrectomy of Polya type with splenectomy was performed There were no secondaries and no evidence of lymphnode involvement The postoperative condition was satisfactory

The resected stomach portion (Fig 4) showed a large carcinoma in the lesser curvature The alkaline phosphatase reaction showed a positive result throughout the entire antrum and parts of the fundal mucosa A positive reaction could also be detected within some parts of the tumour but particularly in its periphery The tumour had penetrated all muscular coats and had infiltrated the subserosa and serosa where an intensely positive alkaline phosphatase reaction could be observed Histologically the tumour was an adenocarcinoma composed of cells of intestinal type with prominent striated borders

The described observations and similar findings in numerous other specimens made us ask ourselves what conclusions, if any, could be drawn First, a few remarks about the ulcer cases The sites of ulcers always bore a close relationship to the advancing edge of the intestinal metaplasia and the ulcers were often surrounded by a zone of intestinal metaplasia Whenever the ulcers were situated high up in the lesser curvature the intestinal metaplasia reached up to the ulcer Intestinal metaplasia is always associated with chronic gastritis The first suggestion put forward is that gastritic mucosa, and in particular the gastritic mucosa with intestinal metaplasia, is a mucosa prone to ulceration Secondly, it seemed more than likely that chr gastritis with metaplasia is a primary condition, in which ulceration occurs as a secondary phenomenon Similar observations and ideas have previously been expressed by *Konjetzny* in his publications

Thirdly, whenever, as in cases of high peptic ulceration, we saw widespread gastritis and intestinal metaplasia we found on clinical investigations a reduced acid secretion or more commonly achlorhydria How can one—under these circumstances—accept the acid secretion as the most essential aetiological factor for peptic ulceration It appeared more likely that an imbalance exists between the protective mechanism of the mucosa—in our case the covering mucus—and the acid produced After all, the mucus produced by the intestinal metaplasia is an acid

mucopolysaccharide with fundamentally different chemical and therefore protective properties from the normal mucus of the stomach which is a mucoprotein The ideas put forward are as yet hypothesis and need further investigation We have attempted to study erosions histochemically in the hope of finding in them small areas with abnormal mucus secretion but owing to the nature of the lesion we have failed so far

Turning to the gastric carcinoma and their association with intestinal metaplasia the findings both histological and macroscopical leave no doubt about the relationship of intestinal metaplasia and cancer in many cases We are however at the present moment not able to decide when an intestinal metaplasia is benign and when it is precancerous Yet it is almost certain that a mucosal change must exist the morphology of which corresponds to the carcinoma *in situ* of other sites Careful histological investigations histochemical studies as well as DNA measurements and chromosomal studies may possibly help to elucidate this gap in our knowledge

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The Department of Histology, University of Uppsala, Sweden

A CELL DISPERSING LIVER EXTRACT INFLUENCING BLOOD CLOTTING

By

K. E. Fichtelius and Birgitta Kullgren

Received 27.ii.65

Influence of Liver Extract on Suspensions of Thymus Cells

The number of cells per cmm in a suspension of thymus or lymph node cells in Earl's solution increased after passage through glass beads coated with water or saline extracts of liver (*Fichtelius* 1964). After the addition of cell-free liver extract to such suspensions the number of cells per cmm was more than 200 per cent of the number of cells per cmm after the addition of Earl's solution. This effect of the liver extract occurred immediately. Experiments with pipettes of different diameters support the view that the liver factor contributes to the dissolution of cell aggregates in the suspensions. It was concluded that the addition of liver extract may be useful as a method by which to obtain the correct cell number in suspensions of lymphoid cells and to estimate the degree of aggregation in such suspensions.

Similar liver extracts are the subject of this article. Our first question was: Is liver extract more effective than other organ extracts in splitting aggregates of lymphoid cells or in preventing the formation of aggregates of these cells?

FORMATION OF AGGREGATES

Experiment 1 A

Materials and Methods

Homogenates of liver, spleen, kidneys, small intestine, lungs, muscles, testes (one part of the organs and two parts of Earl's solution) were prepared from guinea pigs weighing approximately 200 g. The homogenates were centrifuged for 20 minutes at 2500 g and the supernatant fluids were the extracts. They were kept at 4° C and used not later than 72 hours after preparation. Serum was diluted with two parts of Earl's solution, stored and used in the same way as the extracts.

Thymus suspension was prepared as follows. The thymus from a guinea pig was roughly freed from connective tissue and washed in Earl's solution. The surface was carefully freed from remaining connective tissue and the thymus washed once more. *The organ was cut up finely in 3 ccm of Earl's solution and the pieces of tissue were stirred with a pair of forceps and pressed against the sides of the glass* vessel. Another 3 ccm of Earl's solution was added upon which the tissue mixture was sieved through ordinary muslin.

0.1 ccm of the thymus suspension was mixed with 0.1 ccm of Earl's solution or

Supported by the *Swedish Medical Research Council*.

diluted serum or the different organ extracts in the following order Earl's solution diluted serum extracts of liver spleen kidneys intestine lungs muscles testes and

different tests were performed with the extracts from the various organs

Results

After a few mixings aggregates of cells were observed by the naked eye in the original suspension The size of the aggregates appeared to increase as a function of time This aggregate formation was also obvious from the cell counts In all four experiments carried out, the number of cells was lower in the last mixture with Earl's solution, which was made about 10 minutes later than the first See Table 1

TABLE 1

The Number of Cells per cmm in the Earl's Solution Mixtures

	1	2	3	4
Suspension + Earl 1	18075	17875	14750	12400
Suspension + Earl 2	17425	11500	3950	6300

The cell counts per cmm obtained with the various tissue extracts were quite variable This variability can most probably be accounted for by the time dependency of aggregate formation Nevertheless the liver extract mixture, which was the third to be prepared yielded the highest cell count of all mixtures tested The cell counts were higher in all mixtures with serum or organ extracts than in the Earl's solution mixtures This means that serum and all organ extracts possessed aggregate splitting or aggregate inhibiting activity

SPLITTING OF AGGREGATES

Experiments 1 B

Materials and Methods

Extracts and th[illegible] suspension was [illegible] this time Sol[illegible]

[illegible] fifth [illegible] e num[illegible] 1 and

Results

The results are given in Table 2 It is evident that the variation in cell count is very great in this experiment also, probably due to great difficulties involved in the standardization of the shaking of the original suspension before mixing

The highest cell count is found in the mixture with liver extract, and

Acta path et microbiol scandinav 65 60 68 1965

The Department of Histology, University of Uppsala Sweden

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By

K E FICHTELIUS and BIRGITTA KULLGREN

Received 27 ii 65

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Similar liver extracts are the subject of this article Our first question was Is liver extract more effective than other organ extracts in splitting aggregates of lymphoid cells or in preventing the formation of aggregates of these cells?

FORMATION OF AGGREGATES

Experiment 1 4

Materials and Methods

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0 1 ccm of the thymus suspension was mixed with 0 1 ccm of Earl s solution or

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Supported by the *Swedish Medical Research Council*

prevented aggregate formation and that the aggregate preventing factor or factors were present in all extracts and the serum

Influence of Liver Extract on Leucocytes and Thrombocytes

Preliminary experiments —The earlier findings (Fichtelius 1964) and the above results gave rise to the theory that a factor is normally formed or stored in the liver which is necessary for the natural suspension of blood cells *Vuktlalo* (1965) has shown that the liver extract prevents the formation of aggregates in suspensions of bone marrow cells The influence of liver extract on the sedimentation rate and cell counts in different mixtures of red cells has been studied by us, the results obtained were inconsistent Leucocytes and thrombocytes in peripheral blood have been studied with greater success, and some preliminary experiments are reported here

LEUCOCYTES

Experiment 2 4

Materials and Methods

Homogenates of liver were prepared and used as in experiment 1, but the liver was human biopsy material The number of cells per cmm (polymorphonuclears and mononuclears) was counted as in experiment 1 Counts were made from

I) A mixture of 0 5 cc of heparinized blood and 0 5 cc of liver extract after rocking for one hour at room temperature

II) A similar mixture of 0 5 cc of heparinized blood and 0 5 cc of Earl s solution

Results

The results are given in Table 4

It is evident from Table 4 that the number of cells, especially of poly-

e mixture with liver extract

The same tendency has been

which are being continued

TABLE 4

The Number of Leucocytes per cmm in Different Mixtures

	Liver extract + blood		Earl s solution + blood	
	Polymorphonuclears	Mononuclears	Polymorphonuclears	Mononuclears
1	2100	1650	1300	950
2	1900	1400	1700	1800
3	2050	1970	1050	1750
4	1410	2030	1275	1925

The difference between the two types of mixtures may be due to differences in the number and sizes of aggregates, but there may also be

all mixtures with the other extracts or serum show higher values than the mixtures with Earl's solution This suggests that the liver extract is the most effective of the extracts as to split aggregates and that the aggregate splitting factor or factors are present in all extracts and serum

TABLE 2

The Relative Number of Cells per cmm in Different Mixtures Experiment 1 B

	1	2	3	4	5	6	Mean
Proportions	0.1+ 0.1	0.1+ 0.1	0.1+ 0.1	0.1+ 0.025	0.1+ 0.010	0.1+ 0.010	
Rocking time	1 h	2 h	4 h	1 h	1 h	1 h	
Susp + Earl	5.4	27.6	16.0	56.3	5.2	8.6	19.9
Susp + serum	64.0	100	81.5	93.7	35.2	93.8	78.0
Susp + liver	100	88.2	100	100	75.5	100	94.0
Susp + spleen	87.2	79.5	93.8	89.0	100	74.8	87.4
Susp + kidneys	86.0	55.9	70.4	88.8	53.8	93.8	74.8
Susp + intestine	90.9	61.4	87.7	75.3	40.0	78.1	72.2
Susp + lungs	57.4	52.8	79.0	80.0	33.4	87.1	65.0
Susp + muscles	63.2	73.2	74.1	77.9	57.9	79.0	70.9
Susp + testes	77.3	38.6	74.1	78.8	61.7	50.0	63.4
Susp + Earl	3.3	33.1	44.4	46.6	24.5	15.7	27.9

PREVENTION OF FORMATION OF AGGREGATES

Experiment 1 C

Materials and Methods

Extracts
exception .
before the s
0.025 cc of Ea s solution of organ extract or of diluted serum was placed in tubes As soon as the cell suspension was made 0.5 cc was quickly added to each tube with a 5 cc pipette The mixture was gently rocked for one hour at room temperature and the number of cells per cmm counted as in experiment 1 The cell counts were converted into relative numbers (the highest count per cmm = 100) Five tests were performed

TABLE 3

The Relative Number of Cells per cmm in Different Mixtures Experiment 1 C

	1	2	3	4	5	Mean
Susp + Earl	48.9	36.9	51.3	46.6	22.0	41.1
Susp + serum	54.7	86.9	45.1	60.2	90.5	67.5
Susp + liver	100	100	100	100	100	100
Susp + spleen	77.4	62.0	64.8	85.0	56.8	69.2
Susp + kidneys	78.0	91.2	68.3	75.9	76.7	78.0
Susp + intestine	82.0	87.3	85.9	76.4	84.0	83.1
Susp + lungs	51.9	50.0	49.5	76.1	39.2	53.3
Susp + muscles	67.2	57.0	71.3	67.1	48.1	62.1
Susp + testes	57.1	83.5	35.5	69.2	74.9	64.0
Susp + Earl	46.9	47.7	32.0	54.8	28.2	41.9

Results

The results are evident from Table 3

In all experiments the highest count was found in the mixture with liver extract, and the lowest cell count was found in the mixture with Earl's solution This indicates that the liver extract most effectively

Corrected figure for blood-Earl's solution mixture = 2 × obtained figure

The blood-Earl's solution mixture shows lower figures than blood, which indicates the presence of aggregates or loss of cells on the glass walls in this mixture The blood-liver extract mixture, shows higher figures than the blood This indicates that there is a certain degree of aggregate formation or less of cells on the glass walls already in the heparinized blood

TABLE 6

Corrected Number of Thrombocytes in Different Mixtures

	Heparinized blood	Blood + liver extract	Blood + Earl s solution
1	244000	396000	127200
2	254000	288000	189600
3	201200	274400	134400
4	182000	329600	72800
5 *	253600	332800	80800
6 *	258800	333600	92000
7 §	206000	87200	43200
8 §	220000	187200	70400
9 §	193600	107200	104000
10	196000	233200	97600
11	223200	221200	65600

* The liver extract was stored at +4° C for 5 days

§ The liver was stored at +4° C for 2 days before the extract was prepared

Influence of Liver Extract on Blood Clot Formation

Preliminary results —The ability of the liver extract to influence the *suspension stability or the* adhesiveness of thrombocytes suggested that it might be involved in blood clot formation The following experiments were designed in order to test this possibility

Materials and Methods

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or [illegible]

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[illegible]

The following combinations of blood and liver extract were tested

1 Blood from NIH New Zealand [illegible]
2 Blood [illegible] tests
3 Blood [illegible]
4 Blood [illegible]
5 Huma[illegible] tests
6 Blood and liver from Swedish farmer s rabbits 2 tests

an increased destruction of cells in Earl's solution as compared to liver extract

THROMBOCYTES

Experiment 2 B

Materials and Methods

Homogenates of liver were prepared as in experiment 2 A from human liver The supernatant after the first centrifugation was centrifuged for 1 hour at 100 000 g The new supernatant obtained after the floating material had been discarded was the extract which was kept at 4° C, and used not later than five days after the first centrifugation

Thrombocytes were counted utilizing phase contrast microscopy in a Spencer bright-line haemocytometer, 200 small squares being counted twice after dilution 1 20 with the following solution Sodium citrate 5 g, urea 20 g sublimate 0 01 g distilled water *ad* 1000 g

Counts were made from

a) Heparinized blood 10 minutes after sampling
b) The liver extract used
c) A mixture of 0 5 cc of heparinized blood and 0 5 cc of liver extract after rocking for 1 hour at room temperature
d) A similar mixture of 0 5 cc of heparinized blood and Earl s solution

Results

The results are given in Table 5

TABLE 5

Number of Thrombocytes per cmm in Different Mixtures

	Heparinized blood	Liver extract	Blood + liver extract	Blood + Earl s solution
1	244000	31600	214000	63600
2	254000	31600	184000	94800
3	201200	31600	153200	67200
4	182000	31600	180800	36400
5 *	253600	71200	205600	40400
6 *	258800	71200	206000	46000
7 §	206000	9600	48400	21600
8 §	220000	9600	98400	35200
9 §	193600	9600	58400	52000
10	196000	2800	118000	28800
11	223200	2800	112000	32800

* The liver extract was stored at +4° C for 5 days
§ The liver was stored at +4° C for 2 days before the extract was prepared

It is evident that the liver extract used was not free from particles, probably bacterias, which might be counted as thrombocytes with the method used The dilution solution and Earl's solution did not contain such particles However, it is quite easy to correct for the presence of such foreign particles Correction for the dilution 1 1 in c and d was made at the same time, the corrected figures are given in Table 6

The correction formulas are

Corrected figure for blood-liver extract mixture = 2 × (obtained figure — $\frac{\textit{liver extract figure})}{2}$

Corrected figure for blood Earl's solution mixture = 2 × obtained figure

The blood Earl's solution mixture shows lower figures than blood, which indicates the presence of aggregates or loss of cells on the glass walls in this mixture. The blood liver extract mixture, shows higher figures than the blood. This indicates that there is a certain degree of aggregate formation or less of cells on the glass walls already in the heparinized blood.

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Corrected Number of Thrombocytes in Different Mixtures

	Heparinized blood	Blood + liver extract	Blood + Earl's solution
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Influence of Liver Extract on Blood Clot Formation

Preliminary results.—The ability of the liver extract to influence the suspension stability or the adhesiveness of thrombocytes suggested that it might be involved in blood clot formation. The following experiments were designed in order to test this possibility.

Materials and Methods

Whole blood from different species was mixed with equal parts of Earl's solution or with liver extract from different species. The liver extract was prepared as in experiment 1. The mixtures were gently rocked for one hour at room temperat[illegible] Smears were prepared [illegible]

[illegible] erythrocyte [illegible]

embedded and [illegible]

The sections [illegible]

[illegible] xtract were tested

1 [illegible]

2 [illegible] tests

3

4

5

6 [illegible] ver from Swedish farmer's rabbits 2 tests



Fig 1
Blood from guinea pig
To the right Clot formed by rocking with guinea pig liver extract
To the left Clot formed by rocking with Earl's solution

Results

In three of the combinations, 1, 2, and 4, there was a clearcut difference between the clots in all tests, the clot in the mixture with Earl's solution was an ordinary clot containing numerous red cells, but in the mixture with liver extract the clot was small and pale containing relatively few red cells See Fig 1 Histological examination revealed that the pale small clot obtained with liver extract in combination 1 was a fibrin clot resembling the clot at defibrination See Fig 2 The smears from these combinations (1, 2, and 4) did not reveal differences in the size of red cell aggregates in the mixtures with liver extract and those with Earl's solution

In the other combinations, 3, 5, and 6, there were no similar differences between the clots

GENERAL DISCUSSION

It is of very limited value to discuss this liver factor or these liver factors at this very preliminary stage It is sufficient to say that something of great biological interest may be hidden here The factor pre

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Fig 2
Blood from NIH New Zealand Albino rabbit Magnification 175 ×
a Clot formed by rocking with guinea pig liver extract
b Clot formed by rocking with Earl's solution
c Clot formed by shaking with Earl's solution (defibrination)



Fig 2

venting the thrombocyte aggregation may also be of clinical interest. O'Brian (1964) writes in an editorial in the Blood: "However, if one could prevent the platelet mass from forming, it is possible that thrombosis would not occur". And his last sentence: "Evidently it will be difficult to find a therapeutically acceptable inhibitor, but it might be very worth while". We think it could be worth while to continue examinations of the biological effect of the liver extract and try to ascertain from a chemical point of view the nature of the liver factor or factors.

SUMMARY

In an artificial suspension of thymus cells in Earl's solution aggregates of cells are formed during the first minutes of incubation (experiment I A).

The aggregates can be split by adding extracts from the liver and from some other organs, the liver extract being the most effective as regards the splitting of aggregates (experiment I B).

The liver extract was also the most effective as regards the prevention of aggregate formation (experiment I C).

Preliminary experiments are reported which show that liver extract may also prevent the formation of aggregates of blood leucocytes and thrombocytes (experiment II A and II B).

The liver extract also seems to influence the trapping of red cells in a blood clot (experiment III).

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The Tuberculosis and Statistical Departments Statens Seruminstitut Department of Pathology Finsen Institute Central Tuberculosis Dispensary Copenhagen Denmark

HAS ISONICOTINIC ACID HYDRAZIDE (INH) AN ONCOGENIC EFFECT?

By

H C ENGBÆK M WEIS BENTZON H HEEGAARD and O CHRISTENSEN

Received 25 iii 65

A relationship between INH treatment and cancer of the lung was postulated already in 1952 following the observation of extensive metastasis from a bronchial cancer in a patient who because of suspected tuberculosis had been treated with large doses of INH (2) Since then considerable experimental work has been carried out with the aim of elucidating the possible carcinogenic effect of INH Since the administration of INH forms part of the primary treatment of almost all tuberculosis patients and the drug often is given for long periods research in this field is of the utmost importance

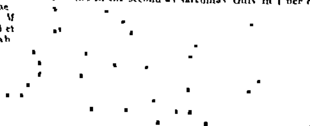
The previous studies can be classified into the following groups

I Has INH a tumour producing effect in normal animals?
II Has INH any effect on the development of implanted tumours?
III Does INH provoke changes in the respiratory system of patients and normal experimental animals i e changes which can be regarded as the first stages in the development of malign tumour?
IV Evaluation of the clinical material

I (a *A tumour producing effect of INH in normal experimental animals* is confirmed from five different sources —

Juhasz et al (11 12) injected INH intraperitoneally into white mice in total doses (8? mg Hungarian preparation) and 55 mg (German preparation) and observed them for 7½ and about 13 months respectively Tumours developed in about 30 per cent (29 out of 95) of the treated animals in the first series mainly as papillary lung adenomas and in the second as sarcomas Only in 1 per cent (1 out of 100 [illegible]

the [illegible]



M [illegible]
d et [illegible]
wh [illegible]

Biancifiori et al (4) administered a total dose of 502 mg INH in water perorally to female Balb/c mice during a period of 46 weeks All of the 38 animals which survived the treatment had tumours of the lungs while 166 controls with similar survival time had none

Weinstein et al (33) carried out their experiments with mice known to have a very low spontaneous incidence of tumours of the lungs (female Black C 57 4 to 6 weeks old) The INH was administered in the food in concentrations from 0 2 to 0 025 per cent during an observation period of 32 weeks None of the controls developed tumour while adenomas were found in the treated animals and a partial dose incidence relationship was present

Schwan (23) injected INH intraperitoneally daily in saline to 6 month old female R 3 mice in a total dose of 109 mg during the course of 87 days Seventeen of the 45 treated animals had tumours of the carcinoid adenoma or papillomatous type and in addition there were two cases of leukaemia No pathological processes could be seen in the 45 control animals

The five above mentioned studies which were performed in Hungary Japan Italy United States of America and Poland respectively showed that the frequency of tumour was greater in treated animals than in those not given INH whether the INH was administered as part of the diet in drinking water or injected subcutaneously or intraperitoneally Furthermore a direct relationship was demonstrated *between size of dose length of treatment and frequency of tumour Such an effect* can scarcely be due to impurities in the drug preparations *Mori et al* (17) attribute significance to the carbamyl group and *Biancifiori et al* (4) to the liberation of hydrazone

I (b) *Reports in which it was impossible to demonstrate any tumour producing effect of INH*

Vallier et al (30) injected white mice (Swiss) intraperitoneally or subcutaneously with large doses of INH twice weekly None of the treated animals which were sacrificed after 5 6 and 7 months showed tumours and there were no cases of leukaemia Neither had aerosol treatment and INH in drinking water any effect

Hackmann (6) examined Neoteben under experimental conditions similar to those used by Juhasz and was unable to demonstrate any increase in the frequency of tumours

Hecht (8) arrived at a similar conclusion in his experiments on rats

In addition to the above mentioned experiments the direct aim of which was to examine the frequency of tumour development other studies have been reported in which INH had been used in animal experiments for long periods and in large doses without any mention of tumour development (10 35 27)

II Experiments concerning *the effect of INH on implanted tumours* have also given varying results

(a) *Enhancing effect*

Tiboldi et al (29) examined Brown Pearce carcinoma in rabbits during treatment with INH (10 mg/kg daily administered by stomach tube for 17 days) and found more extensive metastasis in these animals than in a control group

Wagner et al (3[illegible]
carcinomas in albino rats found that small doses of INH promoted the growth of tumours This was also possible to demonstrate *in vitro* using low concentrations of INH

(b) *No effect*

Epithelial carcinomas induced on the ears of rabbits by means of painting with terpentine were not affected by INH treatment (3)

Tiboldi et al (29) examined Ehrlich ascites tumours in mice as well as Brown Pearce carcinomas in rats without being able to demonstrate any effect of INH

Siegel et al (25 26) also examined Ehrlich ascites carcinoma in mice and could not demonstrate any effect of INH when the treatment was commenced at the time of or after transplantation

Crocker s sarcoma transplanted into mice was not affected by INH treatment (23)

(c) Inhibitory effect

Cancer of the liver was induced by feeding albino rats with DAB (p-dimethyl amino azobenzene) but was inhibited if INH was administered simultaneously in food (14)

Siegel et al (25, 26) considered it of importance that INH should be given as prophylaxis in order to achieve both inhibition of growth and less metastasis after implantation into mice of Ehrlich ascites carcinoma

Finally, *Wagner et al* (31, 32) showed that large doses of INH inhibited the tumours they used, both *in vivo* and *in vitro*

III *Hein et al* (9) *Pagel et al* (18), *Struwe* (28) and *Balo et al* (1) carried out *patho anatomical studies* concerning the bronchial mucous membranes and cavities of patients treated with INH and Ponsa et al (19, 20) similar studies on normal

never in his own laboratory seen any pre cancerous processes in lung resection specimens from patients under intensive treatment with chemotherapeutics, particularly INH

... on that basis rather than as the result of a direct carcinogenic effect of INH

It will thus be seen from the studies mentioned above that experiments with INH have given very divergent results

MATERIAL AND METHODS

... strains were as follows —

The mice were kept separately during the experimental period in 1½-litre *stone* jars The *diet* consisted of a mixture of ...

... *nistration of INH* A preliminary experiment showed that the *Call mice* tolerated a single dose of *1 mg INH* but not 3 mg The other two strains were apparently able to tolerate the larger *dose* Three injections were given each week, *viz 30* per group distributed over a period of about three *months* but with no injections in the second month

* These animals belong to the voles

The doses of INH were as follows —

Strain	Single dose	Total dose
C_1 albino	3 and 0 3 mg	90 and 9 mg
C_3H	1 and 0 3 mg	30 and 9 mg
Red	3 and 0 3 mg	90 and 9 mg

The injections were given intraperitoneally, always in a volume of 1 ml using a fresh needle for each animal

Observation Each animal was observed twice daily and the time of death was recorded Dead animals were sent immediately to the Department of Pathology of the Finsen Institute for autopsy and histological examination If not possible the dead mice were placed in the refrigerator at 4 to 5° C The observation period was about two years as from the last injection, after which all the surviving mice were sacrificed

Autopsy This was carried out in the usual way and any macroscopically visible tumours were described Specimens for microscopy were removed from the tumours and also from the lungs liver, spleen and kidneys of all the animals These specimens were examined together with the routine material of the department and stained in the usual way by the haematoxylin eosin and van Gieson methods The pathologist did not know to which group the mice belonged

Recording of results On a patho anatomical basis the tumours could be divided into three groups —

I Subcutaneous malign tumours, the majority of which were small-celled anaplastic carcinomas usually with a tendency to adenomatous structure a few were sarcomas The histological results do not permit any definite conclusions regarding the histogenesis of the tumours but probably they arose from mammary tissue Five showed metastasis to liver and lungs

II Tumours in the lungs, chiefly adenomas, but also a few carcinomas

III A heterogeneous group of benign tumours, mostly haemangiomas in the liver

RESULTS

Survival times The chances of observing a possible tumour-provoking effect of INH treatment are dependent on the survival times in the different groups Analysis of this aspect is shown in Figs 1a, 1b, 2a 2b and 3 The material is divided up according to INH dosage, and two strains also according to sex (C_1, C_3H) The curves show the percentage number of survivors in months after the first injection In this evaluation the occurrence of tumours was not taken into consideration

All the curves are almost linear from the time of the first death until the end of the observation period

With the exception of the two cases of early death (third and fourth months) in the C_1 control group, the distribution according to survival time can be described as a normal distribution in which some of the values are recorded *only as being greater than the observation period i e* a so-called censored distribution (7) The observation period was 28 months However, as regards the C_3H mice, there was an accumulation of deaths at the end of that period, thus indicating that some special circumstances must have been involved (see Discussion) For those mice, the observation period is therefore taken as 26 months

It can also be seen from the figures that the first deaths in the three strains of mice occurred at different times—the C_1 group (Fig 1) after 8 months (the two early deaths being excluded) and the C_3H group after

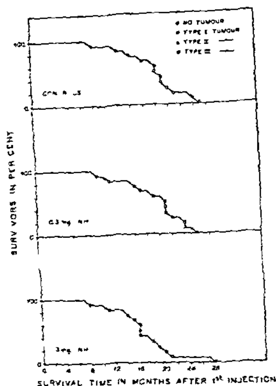


Fig 1a

Survival curves C_1 mice Females

TABLE 1

C_1 Mice

Group	Sex	No of animals	Average	SE	SD
Controls	Female	12	19.8	1.4	4.8
	Male	11*	19.6	1.3	3.7*
0.3 mg INH	Female	12	19.4	1.4	4.9
	Male	13	19.3	1.3	5.1
3.0 mg INH	Female	12	16.8	1.4	5.1
	Male	13	20.6	1.3	5.0
Average	Females		18.7		4.9
	Males		19.8		4.6
	Average		19.3		4.8

* Two mice which died 3 and 4 months after first injection not included in table

4 months (Fig 2) Among the red mice, however, the deaths occurred from the beginning of the observation period (Fig 3)

The calculation technique used in the present work is as described by *Hall* (7)

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Strain	*Single dose*	*Total dose*
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The injections were given intraperitoneally, always in a volume of 1 ml using a fresh needle for each animal

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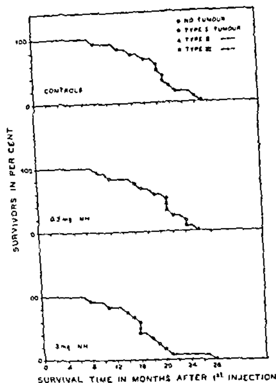


Fig 1 a

Survival curves C_1 mice Females

TABLE 3

C_1 Mice

Group	Sex	No of animals	Average	SE	SD
Controls	Female	12	19 8	1 4	4 8
	Male	11*	19 6	1.3	3 7*
0 3 mg INH	Female	12	19 4	1 4	4 9
	Male	13	19 3	1 3	5 1
3 0 mg INH	Female	12	16 8	1 4	5 1
	Male	13	20 6	1 3	5 0
Average	Females		18 7		4 9
	Males	–	19 8		4 6
	Average		19.3	–	4 8

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4 months (Fig 2) Among the red mice, however, the deaths occurred from the beginning of the observation period (Fig 3)

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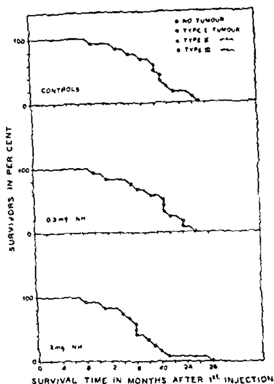


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	Average		*19.3*	–	*4.8*

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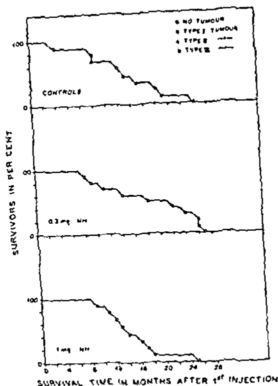


Fig 2 *a*

Survival curves C3H mice Females

TABLE 3

Red Mice

Group	No of animals	Average	SE	SD
Controls	25	15 3	2 4	11 9
0 3 mg INH	25	22 2	3 2	14 7
3 0 mg INH	25	17 0	2 5	12 2
Average		*18 0*	–	*12 9*

males The lowest average survival time (16 8) is seen in the females treated with the largest dose of INH As regards the C3H mice (Table 2), the average survival time is 21 2 and the standard deviation 7 1 Here again there is no difference between the control group and the two treated groups However, distribution according to sex shows that the survival times are considerably shorter for the females than for the males in the control group and in the group treated with the largest dose of INH As regards the *red mice* (Table 3), the average survival time is 18 0 and the standard deviation 12 9, with no dependence on dosage

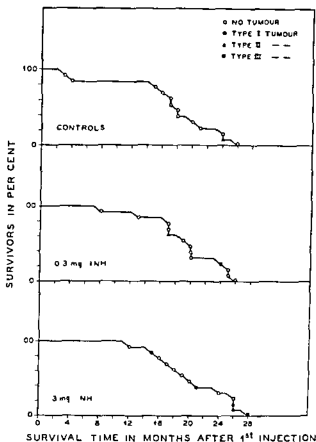


Fig 1 b
Survival curves C_1 mice Males

TABLE 2
C_3H Mice

Group	Sex	No of animals	Average	SE	SD
Controls	Female	9	15 5	2 4	7 0
	Male	16	26 2	2 2	9 6
0 3 mg INH	Female	10	20 1	2 4	9 4
	Male	15	21 5	2 0	7 8
3 0 mg INH	Female	9	15 0	2 4	4 7
	Male	16	23 3	1 9	5 8
Average	Females		16 9		7 0
	Males		23 7		7 7
	Average		*21 2*		*7 1*

Average survival time and standard deviation For the C_1 mice (Table 1) the average survival time is 19 3 months and the standard deviation 4 8 months Neither of the two treated groups deviates from the control group and there is no difference in the survival times for males and fe-

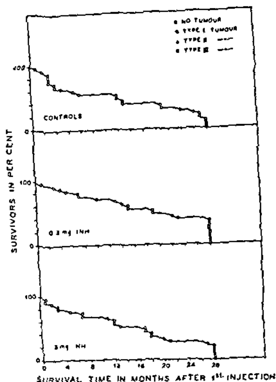


Fig 3
Survival curves Female and male red mice

TABLE 4
Type I and II Tumours in C_1 and C3H Mice
(Dependence on Sex of Animals and INH Treatment)

Sex	Dose of INH	No of animals	
		Total	With tumour
Female	Control	21	10
	Lowest	22	6
	Highest	21	12
Male	Control	29	2
	Lowest	28	1
	Highest	29	9

justifiable to treat the material from the two mouse strains together Comparison is made for each sex between the untreated control animals, the mice treated with the lowest dose of INH (0 3 mg), and those treated with the largest doses (1 or 3 mg) Since separate analysis of the groups with tumour Types I and II showed mainly the same results, these two types are taken together in the present evaluation (Table 4)

It will be seen from Table 4 that there is a high spontaneous incid-

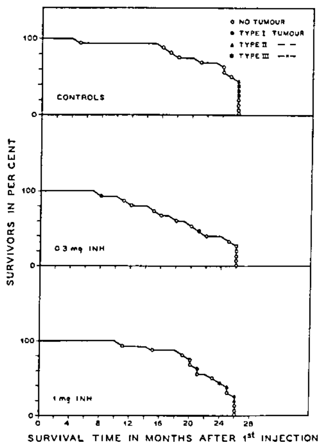


Fig 2 b
Survival curves C_3H mice Males

It was thus found that the average survival time is about the same in the three strains, whereas the standard deviation varies considerably From the point of view of dosage there is no difference, but in certain groups the survival times are found to be shorter for the females than for the males The red mice were not sorted according to sex and this may account for part of the large standard deviation

Occurrence of tumours In Figs 1–3 various symbols are used to indicate in which mice tumours occurred and to which type such tumours belong Almost all the tumours were macroscopically visible

Among the 75 red mice included in the study, tumour was observed in one case only This was a Type III tumour, *i e* belonging to the mixed group of benign tumours Since tumours are rare in this strain, the red mice are not included in the evaluation of tumour frequency

The figures also show that there is no connection between dosage and the occurrence of Type III tumours in the whole material

Thus, tumours of Types I and II in C_1 and C_3H mice form the material used for an evaluation of the frequency of tumours in relation to the sex of the animals and the doses of INH employed

If the individual groups are divided into males and females, it is

TABLE 6

Survival Times in Months from Time of First Injection

Type of tumour	Sex	Mouse strain	Survival times of individual mice: Controls	Dose of INH: Lowest	Dose of INH: Highest	Number	Average	SE
Type I	Female	C_1	10 14 16, 20 21	10 22	15, 16 20	10	16.4*	1.2
		C_3H	15 17 21	11, 18	11, 13 14, 16 17 18	11	15.5*	1.2
	Male	C_1		–	15, 21 28	3	21.3§	2.3
		C_3H		–	21	1	21.0§	3.9
Type II	Female	C_1	18	26	14, 16, 28	5	20.4	–
		C_3H	≥ 26	≥ 26	–	2	≥ 26.0	–
	Male	C_1	24	17	26 26	4	23.3	–
		C_3H	≥ 26		25, ≥ 26, ≥ 26	4	≥ 25.8	–
Type III	Female	C_1	–	16		1	16.0	–
		C_3H	–	≥ 26	–	1	≥ 26.0	–
	Male	C_1	–	24	–	1	24.0	–
		C_3H	≥ 26 ≥ 26, ≥ 26	8 21	24	6	≥ 21.9	–

* Average 16.0, SE 0.9 § Average 21.3 SE 2.0

ence of these tumours (about 45 per cent) among the *female* mice This is not influenced by the doses of INH used The corresponding value for the *male* mice is much lower (about 7 per cent) and is not affected by the lowest dose of INH (4 per cent) However, with the largest dose of INH the incidence rate rises to 31 per cent This is a significant change ($P < 1$ per cent) in comparison with the other two groups taken together

Calculation of survival times, taking the occurrence of tumours into account In order to examine whether INH treatment may cause a more rapid development of tumour, the survival times were further analysed In the subsequent calculations the survival times are divided according to type of tumour (I, II and III), sex of the animals, and INH dosage The red mice are not included

TABLE 5

Average Survival Times of Mice without Tumour

Strain	Dose of INH	Females No of animals	Females Average survival time	Females SE	Males No of animals	Males Average survival time	Males SE
C_1 mice	Control*	6	23.2	1.8	10	19.2	1.4
	0.3 mg	8	19.9	1.5	11	19.1	1.3
	3.0 mg	6	15.5	1.8	8	19.0	1.5
	Total	20	19.6	1.0	29	19.1	0.8
C_3H mice	*Control*	5	11.6	3.3	12	23.0	2.4
	0.3 mg	6	17.9	3.1	13	22.8	2.3
	1.0 mg	3	15.3	4.3	11	22.1	2.4
	Total	14	15.0	2.0	36	22.6	1.4

* Two mice which died 3 and 4 months after injection not included

The results obtained in the mice *in which no tumours could be demonstrated* (Table 5) are generally the same as those obtained in the total material (Tables 1 and 2) The short survival time of female C_1 mice treated with the largest dose of INH deviates significantly from the controls ($23.2 - 15.5 = 7.7$ $SE = 2.5$, $P = 0.2$ per cent) This short survival time may be due to the fact that the female mice could not tolerate the largest dose of INH

The results obtained in the animals *which had tumours at the time of death* are shown in Table 6 In addition to the averages the survival times of the individual animals are stated It will be seen from the table that the dosage of INH has no demonstrable influence on the survival times However, there is a relationship between the survival times and the type of tumour

As regards the mice with *Type I tumours*, no difference can be found between the survival times in the two strains, taking into account the

INH This may possibly explain why studies with similar doses of INH in mice have given such varying results On the basis of the present work a subsequent experiment will be carried out using male C_1 mice only Though INH has an influence on the frequency of tumours it is of no significance for the speed with which the tumours develop

Only one animal had more than one type of tumour (I and II), i.e. a male C_1 mouse treated with 3 mg of INH This animal is included in the Type I group It must be considered unlikely that the malign Type I tumours heal spontaneously If this type of tumour were without influence on the survival time of the animals the frequency of cases with Type I tumours might be expected to increase with the length of the observation period However, there were remarkably few such animals among the mice which had died by the end of the observation period For instance 47 females died between the tenth and twenty fourth month and 21 of these had Type I tumour There were no Type I tumours among the 12 females which died after that time These results seem to indicate that the Type I tumours precipitate the death of the animals The Type II and III tumours were found chiefly among the mice which died at a late stage or which survived the observation period

The present study shows that under certain conditions it is possible to demonstrate an increased development of tumours (carcinomas and lung adenomas) after treatment with INH in total doses not exceeding those used in the treatment of humans when weight is taken into consideration However in this study the drug was administered for a shorter period

Despite these findings and similar results from other quarters INH must continue to play an important rôle in the treatment of patients with tuberculosis Nevertheless the various reports of an increased frequency of tumours should be borne in mind when the use of INH in mass prophylaxis is considered

SUMMARY

A study of the oncogenic effect of INH has been carried out on three breeds of mice C_1 albino CaH black and red (Clethrionomys g Glareolus Schreb) with approximately equal numbers of males and females in each group INH was injected intraperitoneally in the course of about three months the lowest total dose being 9 mg and the highest 30 90 mg The observation time was two years from the time of the last injection

The tumours found were divided on a patho anatomical basis into three categories

I Subcutaneous malignant tumours probably originating in mammary tissue

sex of the animals The average survival times of the females are 16 0, SE = 0 9, and of the males 21 3, SE = 2 0 It is permissible to treat the females of the two strains as one group because the survival times of the female C_1 mice with Type I tumour are shorter than the survival times of the mice without tumours As regards the males, the survival times are the same as in the animals without tumour

On the whole, the animals with Type II and III tumours survived for the longest time

DISCUSSION

The survey of the literature shows varying results as regards the tumour-producing effect of INH Since the animals employed might be of importance in such experiments, three strains of mice were selected for the present study The spontaneous incidence was known for only one of the strains, *viz* the C_3H mice Here the incidence is known to be high, though only among the females Otherwise the experimental conditions in the present study were generally as the ones described by *Juhasz et al* (11)

The tolerance of the three strains to INH differs C_3H mice are the most sensitive, and for that reason the maximum single dose was only 1 mg The C_1 mice are less sensitive, but the female mice seem to be affected by the largest dose of INH (3 mg) The red mice seem to be completely unaffected by that dose of the drug

The curves for the survival times show that it has been possible to run the experiment for a relatively long time without interference by intercurrent disease and consequent incidental accumulation of deaths In July 1962 it was seen how uncontrollable factors can influence the experimental results Within five days 21 C_3H mice died, 20 within two days However, as that episode occurred at the end of the observation period when the experiment with the red mice was concluded and only a few C_1 mice remained, the evaluation of the material was not affected to any critical extent On autopsy the mice showed no signs of infection No explanation can be found why the deaths occurred, but the animals are known to be very sensitive to irregularities in the administration of food and fluids

All the mice were of the same age at the commencement of the experiment The curves show that there is a difference between the three strains as regards distribution of the survival times and that the survival times of the female C_3H mice were shorter than those of the males

The frequency of tumours in the three strains shows clearly that it is important to choose the correct experimental animals The ability of INH to provoke tumours (Types I and II) was not found in the red mice with their very low spontaneous incidence of tumour nor in the female C_1 and C_3H mice with a high spontaneous incidence Only the corresponding males with a low incidence showed a significant increase in the number of tumours in the group treated with the largest dose of

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II Tumours in the lung, chiefly adenomas but also a few carcinomas

III A heterogeneous group of benign tumours

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distribution are most often seen (2, 3). A few examples of pock counting techniques with a variation approaching the lower limit of Poisson variability have however been described (2, 7). In these studies meticulous care was taken in the handling of the eggs employed.

The problem why bacterial countings not uncommonly show a variation exceeding that expected from the Poisson distribution while plaque countings of infectious virus particles, at least according to our experience, often show Poisson variation will not be discussed in detail in the present paper. It should be mentioned however, that the mode of reproduction, the surface characteristics and the small particle size of most viruses are some of the factors which most likely play a role for the tendency of virus particles to show Poisson distributions.

The fact that plaque titrations are more likely to show Poisson variation than the pock titrations are seems to be due to certain technical difficulties inherent in the pock titration technique, namely the individual variation in eggs in membrane sensitivity for viruses, variations in the amount of inoculum actually reaching the chorioallantoic membrane and the production of secondary pocks (2). These factors which tend to decrease the accuracy of the pock titration techniques are avoided when an appropriate plaque titration technique is employed.

The Poisson distribution is defined by the following formula expressing that the probability of counting x particles is

(1.) $$p\{x\} = e^{-\lambda}\,\lambda^x/x!$$

where x theoretically may have all values from zero and upwards. λ is the expected "true value", i.e. the expected number of particles calculated from the particle concentration of the original suspension and the volume of the inoculum.

The mean value and the variance of x is

(2.) $$M\{x\} = \lambda$$

(3.) $$V\{x\} = \lambda$$

To test whether certain observations follow the Poisson distribution the following method can be used. If one has a series of countings from the same suspension $x_1, x_2, \ldots, x_n$ the average value $\bar{x}$ and the mean square s^2 is calculated. From (2.) and (3.) it would be expected that the ratio $s^2/\bar{x}$ was close to 1. It can be shown (6) that this ratio with good approximation follows a χ^2/f distribution with n − 1 degrees of freedom so that we have

(4.) $$s^2/\bar{x} \sim \chi^2/f \qquad f = n-1$$

Therefore the fractiles in the χ^2 distribution can be used to estimate whether an observed value of $s^2/\bar{x}$ is unusually high. Investigations (13)

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SOME STATISTICAL PROBLEMS IN RELATION TO THE USE OF THE POISSON DISTRIBUTION IN VIRUS PLAQUE ASSAYS

By

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In a preceding paper details concerning the technique and use of a Sindbis virus plaque assay has been described (11) The present communication is concerned with the methods used and the experiences gained in the statistical analysis of this assay and other virus plaque assays performed in this institute

MATERIALS AND METHODS

The tissue cultures egg techniques and viruses employed in this study were described in detail elsewhere (1 7 11)

Since "Student" in 1907 employed the Poisson distribution for counting of yeast cells under the microscope (12) this distribution has been customarily used to describe distributions of the number of different particles contained in a certain volume or on a certain area On the basis of certain assumptions a Poisson distribution of values obtained in particle countings can be predicted theoretically (2, 6, 8) Shortly these assumptions express that the particles should be independent and not have any tendency, for instance due to a common source of origin or due to heterogenicity of the substrate, to cluster in certain areas

Often the above mentioned assumptions are not fulfilled in countings of bacteria and consequently a variation exceeding that expected from the Poisson distribution may be encountered

Our experience is however, that counting of virus particles may well follow the Poisson distribution This may be the case in virus plaque titrations where a virus suspension is inoculated onto a monolayer of tissue culture cells and the number of infectious virus particles is estimated from the number of plaques produced (11, 1) In pock titrations where a virus suspension is inoculated on the dropped chorioallantoic membrane of an embryonated egg and the number of infectious virus particles is estimated from the number of pocks produced upon the membrane, variations surpassing those to be expected from the Poisson

then the sum $S = x_1 + \quad + x_k$ will be Poisson distributed with the mean value $\lambda = \lambda_1 + \quad + \lambda_k$. It can be shown that all information about λ is contained in S and that once the ratios between $\lambda_1, \lambda_2,$ and λ_k are known knowledge of how the sum S has arisen from the single values renders no additional information. These facts make it preferable to work with sums and not with average values, whenever Poisson distributed values are concerned.

Having observed two Poisson distributed variables with the unknown parameters (mean values) λ_1 and λ_2 it is often desirable to investigate *whether the ratio* λ_1/λ_2 may have a certain value k (for $k = 1$ we get $\lambda_1 = \lambda_2$). Thus we consider the hypothesis

(5) $$\lambda_1/\lambda_2 = k$$

The variable $x_1 - kx_2$ will now, according to the hypothesis, have the mean value 0 and (according to (3)) the variance $\lambda_1 + k^2\lambda_2 = \lambda_1(1 + k)$. This variance is unknown but according to the addition theorem $x_1 + x_2$ can be used as an estimate of $\lambda_1 + \lambda_2 = \lambda_1(1 + 1/k)$. If the x values are not too small we can consider

(6) $$u = \frac{x_1 - kx_2}{\sqrt{k(x_1 + x_2)}}$$

as normally distributed with the mean value 0 and the standard deviation 1. There will for instance—still according to the hypothesis (5)—be a probability of only 5 per cent that the u-value will fall outside the interval from −1.96 to +1.96. Hence an observation falling outside this interval may be considered as a significant deviation from the expectancy. In certain instances it is more appropriate to assume that the u-value with a probability of only 5 per cent should be 1.64 or more.

For small x'es the approximation to the normal distribution is not good. Therefore the special properties of the Poisson distribution has to be utilized. Since the sum $x_1 + x_2$ contains all information about $\lambda_1 + \lambda_2$ the conditional distribution of x_1 for a given sum $x_1 + x_2$ depends only of the value of k. We find the following binomial probability

(7) $$p\{x_1 \mid x_1 + x_2\} = \binom{x_1 + x_2}{x_1}\left(\frac{k}{1 + k}\right)^{x_1}\left(\frac{1}{1 + k}\right)^{x_2}$$

For $k = 1$ this result is well known, but for k different from 1 it appears to be used only rarely.

(7) can be used, most easily by employing a table of the binomial distribution, to estimate whether the observed values of x_1 and x_2 seem reasonable according to the hypothesis (5). For $k = 1$ special tables and diagrams have been worked out (9).

The fact that very high concentrations of virus cannot be used is a complication in plaque assays. With high concentrations of virus

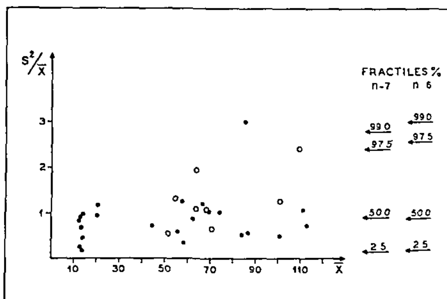


Fig 1
Diagram showing corresponding $s^2/\bar{x}$ and $\bar{x}$ values of a Sindbis virus plaque assay Each point represents an average value of counts obtained from 6 (signature ●) and 7 (signature ○) cultures respectively For further signatures see text

seem to show that this approximation to the χ^2 distribution is good even for relatively small values of n and $\bar{x}$ For small values of n and $\bar{x}$ the methods proposed by *R A Fisher* (5) and further developed by *Radhakrishna Rao & Chakravarti* (4, 10) my be used

However rather large numbers of experimental series each containing n (or a varying number of) cultures all of which have been inoculated with the same virus suspension, are often used for practical purposes

In such cases it is useful to plot the observations in a diagram showing the corresponding values of $s^2/\bar{x}$ and $\bar{x}$ Discrepancies from the Poisson distribution will often be most obvious for high values of x and appear in a systematic way in such a diagram Fig 1 shows an example of this type of diagram where a good agreement between the observations and the Poisson distribution was obtained

An addition theorem which seems rather obvious, but which can also easily be proven mathematically holds for the Poisson distribution (6) If two areas (for instance monolayers of cells) are seeded with x_1 and x_2 particles these two areas may be considered as parts of one big area seeded with $x_1 + x_2$ particles Accordingly the theoretical reasons for Poisson distributions of x_1 and x_2 may also be extended to the sum $x_1 + x_2$ Naturally the two "original" areas need not be of equal size and consequently the two "original" Poisson distributions need not have identical mean values Generally it can be said that if the variables $x_1, \quad, x_k$ are Poisson distributed with the mean values $\lambda_1, \quad, \lambda_k$

then the sum $S = x_1 + \quad + x_k$ will be Poisson distributed with the mean value $\lambda = \lambda_1 + \quad + \lambda_k$. It can be shown that all information about λ is contained in S and that once the ratios between λ_1 λ_2 and λ_k are known knowledge of how the sum S has arisen from the single values renders no additional information. These facts make it preferable to work with sums and not with average values whenever Poisson distributed values are concerned.

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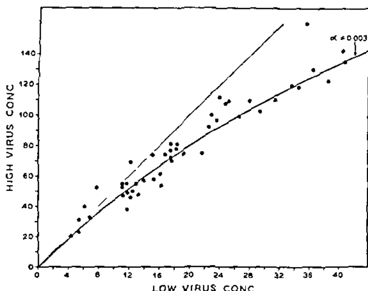


Fig 2

Diagram showing depression of plaque counts occurring when high concentrations of virus are used in a vaccinia virus plaque assay. Each point represents an average value of counts obtained from 5 cultures. The low concentration of virus has in all instances been a 1 : 5 dilution of the high concentration of virus. For signatures see text.

some plaques may be confluent and the use of even higher virus concentrations makes plaque counting impossible. A low concentration of the virus seed allowing exact plaque counts is on the other hand not desirable since this will reduce the relative accuracy of the assay considerably. It is therefore often attempted to employ a virus concentration of "medium" size, hoping that confluence of plaques will not occur to any greater extent. Since it is often impossible to choose in advance a suitable concentration for inoculation because the virus content of the original suspension is unknown, virus plaque titrations are frequently performed by inoculating several cell monolayers with varying dilutions of virus.

Fig 2 shows an example of plaque titrations where two virus suspensions with a 5-fold difference in concentration were employed in each experiment (marked with a point in the figure) (1). Each value is the average value of plaque counts obtained from 5 monolayer cultures.

It can easily be seen that there is no proportionality between the results obtained with the two different concentrations but that the number of plaques is lower than would be expected when the higher virus concentrations are used. To describe this phenomenon the following model can be used

(8.) $$\lambda = \lambda_0/(1 + \alpha \lambda_0)$$

where λ_0 is the actual concentration of infectious virus particles, α is a small figure and λ is the mean value of the Poisson distribution ob-

served when the given concentration is inoculated into cell monolayers and plaque counts are made It can be seen that when λ_0 is increasing λ will increase asymptotically towards the value $^1/\alpha$

With two virus concentrations λ_0 and 5 λ_0 the two mean values $\lambda_1 = \lambda_0/(1 + \alpha\lambda_0)$ and $\lambda_5 = 5\lambda_0/(1 + 5\alpha\lambda_0)$ are obtained according to (8). For varying λ_0 a curve of corresponding values of λ_1 and λ_5 can be made Fig 2 shows this curve for $\alpha = 0.003$ It appears that good agreement exists between the observations and the model (8)

The value 0 003 chosen for α has been found graphically as the value being most compatible with the observations A direct estimation of α is difficult and for small materials hardly of much value It seems most appropriate in using the model (8) to consider α as a value characteristic for the technique employed Knowledge of the magnitude of α must then be obtained from a sufficently extensive experimental material in which the same technique has been used

(8) has been chosen only because it offers a relatively simple description of the depression of plaque counts at higher concentrations However, a certain analogy can be made between the model (8) and theory [illegible]

[illegible] (8) on certain assumptions regarding the dead time

It is obvious that the equation (8) does not hold when λ approaches the asymptote $1/\alpha$ compatible with the fact that complete confluence of plaques will make plaque counting impossible Under such conditions the expression

$$\lambda = \lambda_0 e^{-\alpha\lambda_0} \qquad (9)$$

might be employed instead of (8) (9) has also been worked out by Takacs on the basis of slightly different assumptions regarding the dead time of a Geiger counter These suppositions may be considered as expressive of confluence of the registration of particles When λ_0 is small (9) is not very different from (8) and since one would hesitate to use (8) as well as (9) for values of λ close to the maximum value we have decided to use (8 [illegible]

Considerin[illegible]
with less tha[illegible]
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t[illegible]

[illegible] the technique of counting may create small variations of α for high values of λ_0 This would tend to counteract the possible decrease in variability

If x particles are counted the ordinary maximum likelihood theory together with (8) will give the estimate $x/(1 - \alpha x)$ of λ_0 with an estimated variance of $x/(1 - \alpha x)^4$ However, under conditions where two

estimates of λ_0 can be obtained from two different dilutions of a virus suspension the complication of combining the two estimates arises

Suppose that one of the dilutions is g times more concentrated than the other one and that n monolayer cultures are seeded from each of the two dilutions If S_1 and S_g are the respective sums of the plaques obtained and μ_1 and μ_g the corresponding mean values we have according to (8)

(10)
$$\mu_1 = \frac{n\,\lambda_0}{1 + \alpha\,\lambda_0} \sim S_1$$

$$\mu_2 = \frac{n\,g\,\lambda_0}{1 + \alpha\,g\,\lambda_0} \sim S_g$$

Thus the following probabilities of the observations are obtained

(11)
$$p\,\{S_1, S_g\} = e^{-(\mu_1+\mu_g)}\,\mu_1^{S_1}\,\mu_g^{S_g}/S_1!\,S_g!$$

(12)
$$p\,\{S_1 + S_g\} = e^{-(\mu_1+\mu_g)}\,(\mu_1+\mu_g)^{S_1+S_g}/(S_1+S_g)!$$

$S_1 + S_2$ is not sufficient for the estimation of λ_0 When, however, maximum likelihood estimation is employed (11) renders a fourth degree equation and (12) only a second degree equation for the estimation of λ_0 If the likelihood function is used to determine the asymptotical variance of the estimates, it can furthermore be shown that (11) only renders a slightly better estimate than (12) Even for $\alpha\,\lambda_0$ as high as ½ the variance ratio is only 1 19 It seems reasonable therefore to use (12) in estimating λ_0 The estimate is determined by the equation

(13)
$$\frac{S_1 + S_g}{n} = \frac{\lambda_0}{1 + \alpha\,\lambda_0} - \frac{g\,\lambda_0}{1 + \alpha\,g\,\lambda_0}$$

It has been found convenient to work out a diagram giving λ_0 as a function of $(S_1 + S_2)/n$, depending of course of g and α It is, however, also possible by employing (11) to work out a nomogram from which the estimated values of λ_0 can be obtained (for instance by employing S_1 and S_g as values in a coordinate system and showing curves for the different values of λ_0) By using such a nomogram it would also be possible to exert some control on the consistency of S_1 and S_g

It is not necessary always to consider the depression of plaque counts, occurring when high concentrations of virus is employed The purpose of the investigation is often to see whether two sums of Poisson distributed plaque counts may have the same mean value In titrations of interferon (11) or in virus neutralization tests one may have two inhibition (neutralization) curves corresponding to two batches of interferon or two sera If the same virus concentration, the same interferon dilutions and the same number of monolayers per dilution is employed in the titrations the two sums of plaque counts may be compared according to (6) with $k = 1$ irrespectively of the possible flattening of

the inhibition curves for small interferon concentrations and irrespectively of the shape of these curves

Let us assume that one monolayer culture is used per dilution and that the interferon batches (sera) are employed in the dilutions 1 1, 1 5 1 25 1 125 and 1 625 If the following plaque counts are obtained Batch A 2 30 70 120 and 125 and batch B 5, 40 75 121 and 122 and the sums $S_A = 2 + 30 + 70 + 120 = 222$ and $S_B = 5 + 40 + 75 + 121 = 241$ are compared according to (6) u will have the value 0 88 i e the inhibitory capacity of the two batches is not significantly different

The reason for excluding the counts 125 and 122 obtained with the 1 625 dilutions is that these counts are undoubtedly only little influenced by the presence of interferon and therefore do not expand the knowledge of the inhibitory capacity of the batches On the other hand the counts obtained with the 1 625 dilutions might have been included in S_A and S_B but this would only have weakened the power of the test (6)

It is remarkable that this comparison of two inhibition curves can be carried out without regard to the shape of the curves Often there is no reason to attempt a calculation of 50 per cent inhibition end point titres when the aim of the titration is only to determine whether a difference exists between the inhibitory capacity of two batches of interferon

Calculation of 50 per cent end point titres are however often performed in interferon assays and differences of such titres are employed to expr ... We feel that ... pon a certain

SUMMARY

Methods employed in the statistical analysis of virus plaque assays where Poisson variability of the number of plaques were obtained are described Special reference is given to the difficulties arising when high concentrations of virus are used as *inocula* in virus plaque assays

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CEREBROSPINAL FLUID PROTEINS IN THE GUILLAIN-BARRÉ SYNDROME

Immunological Studies

By

HENRIK RAVN and KLAUS JENSEN

Received 16.i.65

More than a hundred years ago *Landry* (11) as the first reported ten cases of a syndrome, which since 1916 has been named Guillain-Barre's Syndrome (G.B.S.). The names of the French investigators *G. Guillain & A. Barre* (6) were connected with this disease because they had demonstrated that "dissociation albumino-cytologique" in the cerebrospinal fluid (CSF) was a characteristic feature of the Syndrome. Albumino cytological dissociation means elevated CSF protein without pleocytosis. The interest in the disease was greatly stimulated by this discovery giving rise to an overwhelming literature on the subject and several investigations of the aetiology. The aim of these investigations has been partly to demonstrate whether microorganisms might be causative agents of the syndrome, partly to investigate qualitatively the CSF protein, stimulated by the theory that G.B.S. were an autoimmune disease (*Melnick* (15)).

The purpose of the present investigation has been to demonstrate immunological differences between the proteins and other antigenic substances in CSF from patients with G.B.S., compared to those found in normal CSF and serum. To this end the extremely sensitive Schultz Dale technique has been employed. By these investigations it was our hope to reveal a possible causative microorganism or to demonstrate antigenic substances liberated from the nervous tissue indicating autoimmune mechanisms. Supplementary studies using the gel-precipitation reaction have been made simultaneously.

PREVIOUS INVESTIGATIONS

[...] by a grant from *Gårdejer of Stenløse P. L. Pedersens Foundation* and a U.S. Public Health Service Grant No. RF-40

doivent sans doute etre invoquees mais nous n avons pu les deceler Since then numerous attempts have been made at demonstrating causative microorganisms in G B S As early as in 1918 *Bradford Bashford & Wilson* (1) published 30 cases of acute polyradiculitis Spinal cord tissue from the fatal cases was emulsified and injected intracerebrally into monkeys resulting in a development of a similar disease Later on these results have been subject to doubt because they could not be reproduced by others Thus in 1932 *Demme* (3) injected spinal cord tissue from a typical and fatal case of G B S into the brain and sciatic nerve of two monkeys which subsequently remained perfectly well In 1943 *Lassen Ipsen & Bang* (1?) performed several experiments using nervous tissue and CSF from five evident cases of G B S of which four were fatal They injected emulsified tissues and CSF into approximately 200 experimental animals comprising mice rabbits ferrets guinea pigs and monkeys all of which remained healthy

In 1949 *Haymaker & Kernohan* (7) published 50 fatal cases of G B S In 17 cases nervous tissue was examined for virus with negative results In eight cases cultivation of CSF and in seven cases blood culture revealed no microorganisms

Jackson (8) published 28 cases of G B S From two of these cases coxsackie virus was isolated In these two patients however important deviations from the classical G B S were noted and hence the diagnosis must be considered doubtful

Among the 127 patients in Blegdamshospitalet reported by *Rain* (17) 32 cases have been examined by cultivation for polio and coxsackievirus in the CSF in addition 28 of these cases were examined for ECHO virus These examinations have been carried out at Statens Seruminstitut the polio department All these attempts at virus cultivation have been negative Summarizing it can be substantiated that hitherto no attempts at isolation of microorganisms from G B S have been successful

Investigations into the nature of CSF protein in G B S—In 1961 *Spina Franca & Saraira* (18) examined the CSF protein from seven patients with G B S using paper electrophoresis on concentrated CSF They found in all cases that the main part of the increase in CSF protein was caused by globulins

Immunoelectrophoretic studies of CSF by *Clausen Krogsgaard & Quaade* (2) have shown that the proteins found in the CSF from seven patients with G B S could not be qualitatively distinguished from the normal serum proteins and that the increase in the total amount of protein in the CSF in five of these seven patients was caused mainly by an increase in the immunoglobulins

Dencker Swahn & Ursing (4) have made a similar study on seven patients with G B S and found the same changes in the CSF The increase in the immunoglobulins was also found in cases with normal total protein content The increase took place within the first three weeks of the disease and returned to normal values parallel with the disappearance of the symptoms The methods employed by these authors however were not suitable for a demonstration of the presence of small amounts of proteins normally not found in human serum because the protein fractions were traced using horse immuneserum against normal human serumproteins Neither is the immunoelectrophoretic method sufficiently sensitive to demonstrate small amounts of antigens

Laterre et al (13 14) have made a similar study on CSF from patients with various neurological disorders They demonstrated nine different antigenic components not found in normal human serum Only one of these components (post gamma) was studied in detail In none out of eight cases of G B S it was possible to demonstrate this antigen

MATERIAL AND METHODS

Normal Material

Human sera were obtained from healthy adults a control paper electrophoresis showed normal values Normal CSF from patients with no symptoms of G B S was obtained from a neuroradiological department Control examination of the CSF showed no cells and normal protein values Normal nervous tissue originated from a patient who died from a laryngeal carcinoma

Materials from Patients with G B S

Pathological CSF and sera were obtained from six patients fulfilling all the above mentioned criteria of G B S Pathological nervous tissue derived from a fatal case of G B S The patient died after eight days duration of the disease involving rapid

progression of the paralyses The autopsy (*Erna Christensen*, M D) showed polyradiculopathia acuta

Preparation of Antigens

All sera, CSF and nervous tissue preparations were kept sterile at 4° C, and merthiolate was added to a final concentration of 1 : 10 000 CSF was used undiluted Nervous tissue from the spinal cord and spinal roots was used as an emulsion of 1 5 g of tissue per 10 ml saline As adjuvant was used "Freunds Adjuvant Complete, Difco The emulsions of antigens in adjuvant were prepared as water in oil emulsions immediately prior to injection

Sensitization of Experimental Animals

As experimental animals were used 32 guinea pigs They were sensitized by intraperitoneal injections In the first experiment one half of the animals were sensitized without addition of adjuvant, the other half of the animals were sensitized with adjuvant added to the antigens In the following experiments adjuvant was used constantly because it proved to give the best sensitization and no false reactions The

ficient amounts of pathological CSF

TABLE 1

Schedule for Immunization of Guinea Pigs

	1 day	5 day	10 day	20 day	25 day	30 day	45 day
First group	0 1	0 5	1 0	0 5	1 0	1 0	0 5
Following groups	0 2	0 5	0 2	0 5			

The figures indicate ml of CSF serum and nervous tissue emulsions used In most cases equal amounts of complete Freund s adjuvant were added All injections were intraperitoneal

Schultz Dale Technique

The sensitized guinea pigs we
cardiac puncture followed by
after this procedure approxim
removed by cutting the omen ... as possible The removed piece of ileum was washed inside and outside with oxygenated Ringer's solution at 37° C and cut into 2 5 cm segments In order to have the segments attached in the Schultz Dale apparatus small loops of th

histamine as well as
were always add ... xamined,
given by the mo ... onses are
taneous contract ... ess spon
The Schultz D
Faurby Lundbe ... (9) and

Gel D

a

Agar No 2 1 25 per cent The agar was dissolved by heating to 100° C Higher temperatures were avoided as they will cause hydrolysation of the agar 0 85 per cent NaCl and merthiolate 1 10 000 were added and the agar poured into disposable plastic petri dishes After drying the agar plates for one hour, the wells and cups were cut in a pattern as shown in Fig 2 This pattern is well suited for a comparison of several unknown antigen solutions with known antigens (*Jensen* (10))

The gel precipitation technique has the advantage that complex antigen antibody systems can be compared and the precipitation lines in one system identified with precipitation lines in another system by 'reaction of identity' (*Ouchterlony* (16)) In this way the antigens in pathological CSF can be compared with the antigens in normal CSF and normal serum This technique however does not allow the demonstration of small amounts of antigens

RESULTS

Immunization with Normal CSF

a) *Schultz-Dale experiments* —In this study the Schultz-Dale (S D) reaction proved to be equal to its reputation as being very sensitive Occasionally, however, the reproduction of the single reaction failed, not only when repeated in another animal, but also when repeated on another segment from the same animal It was therefore necessary to use many guinea-pigs and to repeat the experiment several times on different segments to strengthen the evidence of the results Hence, if results were not clear-cut they were expressed as number of positive reactions in relation to attempts

The animals were sensitized with a total amount of 0 8 mg of CSF protein, and in all cases (seven animals) S-D reactions could be elicited with 1 ml of normal CSF (0 3 mg protein) A similar reaction could be elicited with pathological CSF and normal human serum and fractions hereof (γ-globulin and albumin)

Subsequently a new series of S-D experiments were performed Initially the segments were rendered refractory to normal CSF by repeated challenge with this antigen Subsequently the same segments were challenged with pathological CSF According to expectation this experiment was negative in five of six cases The single positive reaction can be explained by the observation, that a segment rendered refractory to a certain dose of antigen, still could react with a much bigger dose of the same antigen In the positive case the pathological CSF contained 750 mg of protein per 100 ml, as compared to 27 mg per 100 ml in the normal CSF Similar results could be obtained with diluted and undiluted normal human serum These results were taken into consideration in the following experiments, where the reagents were used in approximately equal concentrations Finally it was shown in animals from this group that segments made refractory to human γ-globulin could react with human albumin , and subsequently, when they were refractory also to this substance, a third reaction could be elicited with normal human serum, (see Fig 1) Similar reactions were elicited with the same antigens in the sequence albumin, γ-globulin, and serum

The results of these experiments are summarized in Table 2

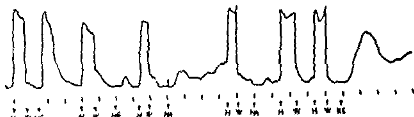


Fig 1

Schultz Dale experiment with ileum from guinea pig sensitized with normal CSF HG = normal human gammaglobulin HA = normal human albumin NS = normal human serum H = histamine chloride 10 µg W = 3 washes with Ringer s solution

TABLE 2

Results of S D Experiments with Ileum from Guinea Pigs Sensitized with Normal CSF

Primary anaphylactic reaction exhausted with	Secondary anaphylactic reaction with				
	NCSF	PCSF	HA	HG	NS
Normal CSF (NCSF)	—	— (5 out of 6)			+ — (1 out of 2)
Pathological CSF (PCSF)		—			
Normal human albumin (HA)			—	+	+
Normal human γ globulin (HG)			+	—	+
Normal human serum (NS)	—		—	—	—

b) *Gel precipitation reactions* Gel-precipitation reactions using serum from guinea pigs sensitized with normal CSF gave no reactions with normal or pathological CSF or human albumin, but with normal human serum two precipitation lines were produced, one of which was identical with the single line produced with human γ-globulin, (see Fig 2)

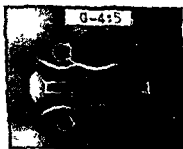


Fig 2

Gel precipitation reaction The fig shows a central long well surrounded by six cups The cups (in this fig and figs 4 and 6) are numbered as follows $\frac{1\ \ 2\ \ 3}{4\ \ 5-6}$ The central well contains serum from guinea pig sensitized with *normal CSF + horse protein* The *cups contain* 1 Normal human gammaglobulin 2 Normal human serum 3 Normal human albumin 4 Normal human gammaglobulin 5 Normal horse serum 6 Normal human albumin

Agar No 2, 1 25 per cent The agar was dissolved by heating to 100° C Higher temperatures were avoided as they will cause hydrolysation of the agar 0 85 per cent NaCl and merthiolate 1 10,000 were added and the agar poured into disposable plastic petri dishes After drying the agar plates for one hour, the wells and cups were cut in a pattern as shown in Fig 2 This pattern is well suited for a comparison of several unknown antigen solutions with known antigens (*Jensen* (10))

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Fig 4

Gel precipitation reaction *Central well* serum from guinea pig sensitized with pathological CSF *Cups* 1 Normal human nervous tissue 2 Normal human serum 3 Pathological human nervous tissue 4 Normal human gammaglobulin 5 Normal horse serum 6 Normal human albumin

quent challenge with the same dilution showed no response This experiment shows that if the ileum is to be made refractory, the desensitizing dose must be the same or greater than the eliciting dose

The results are summarized in Table 3

TABLE 3
Results of S D Experiments with Ileum from Guinea Pigs Sensitized with Pathological CSF

Primary anaphylactic reaction exhausted with	Secondary anaphylactic reaction with			
	NCSF	PCSF	NS	NN
Normal CSF (NCSF)	—	— (13 out of 15)	+ (6 out of 7)	—
Pathological CSF (PCSF)	—	—	— (7 out of 8)	—
Normal human serum (NS)	—	—	—	—
Diluted normal human serum (DNS)	— (3 out of 4)		+ (4 out of 6)	
Normal nervous tissue (NN)	+	+	+	—

b) *Gel precipitation reactions* —Gel precipitation reactions with serum from guinea pigs sensitized with pathological CSF demonstrated antibodies against three to five different antigens, all of which were found in normal human serum (See Fig 4) It was not possible by this method to demonstrate antibodies against antigens specific of pathological CSF or nervous tissue In this connection, however, it must be remembered that gel precipitation is visible only when high concentrations of antigens are used Thus using normal CSF only one faint line was seen, whereas two lines were visible if pathological CSF were used as antigen in this test

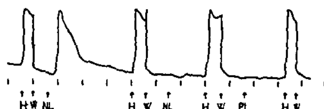


Fig 3

Schultz Dale experiment with ileum from guinea pig sensitized with pathological CSF H & W see Fig 1 NL normal CSF PL = pathological CSF

Immunization with Pathological CSF

a) *Schultz-Dale experiments* —This group consisted of 15 guinea pigs In all cases typical S-D reactions were provoked with normal and pathological CSF, normal and pathological human serum, normal and pathological nervous tissue

As regards this group the most important question was whether S D reaction could be elicited by pathological CSF after the ileum had been made refractory to normal CSF This experiment was repeated 15 times In 13 of these the results were negative (see Fig 3), in the two positive experiments, the protein concentration in the pathological CSF exceeded considerably the concentration in the normal CSF (722 mg %/27 mg %) Analogous results were obtained using undiluted human serum as the second stimulus

Another important question was whether it might be possible to elicit a S-D reaction with pathological CSF after the ileum had been made refractory to undiluted normal human serum This was attempted 10 times, the results being negative every time When the ileum was made refractory with normal human serum diluted to the same protein concentration as the pathological CSF, a subsequent positive reaction could be elicited with pathological CSF in four out of seven cases In these four cases, however, no primary S-D reaction was obtained with the diluted human serum

In an attempt to learn whether the positive S-D reaction elicited with extract of normal nervous tissue might be due to the presence in pathological CSF of nervous tissue antigens not normally present in CSF, it was tried to elicit this reaction on ileum made refractory to normal CSF This proved to be impossible If used in inverse order, however, normal CSF was always able to give positive reaction after exhaustion of the reaction with nervous tissue

To evaluate the few instances in which a positive secondary reaction was obtained with pathological CSF after desensitization with normal CSF, the *quantitative relationship between desensitizing and eliciting* doses was studied Ileum from a sensitized guinea-pig was challenged with normal human serum in the concentrations 1 10 000, 1 1 000, 1 150, 1 100, 1 10, and undiluted serum in this sequence With each dilution a weak *positive reaction* was obtained, whereupon a subse

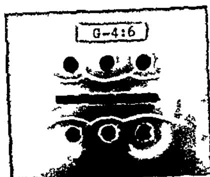


Fig 6

Gel precipitation reaction *Central well* serum from guinea pig sensitized with normal CSF + diluted horse serum *Cups* 1: Normal horse serum 2 Normal human serum 3 Normal human gammaglobulin 4 Normal horse serum 5 Normal human gammaglobulin 6 Normal human serum

with the same antigen showed that the ileum was made refractory, whereupon a challenge with horse serum gave another anaphylactic contraction (see Fig 5) This secondary reaction could be elicited with 50 µg of horse protein (= 2.5 µg/ml) Using horse protein as the first stimulus a contraction could be obtained with 0.5 µg of horse protein (this amount of protein is diluted in the reaction chamber with approximately 20 cc of Ringer's solution which gives a final concentration of 25 nanogram of protein per ml) Using the antigens in reverse order similar results were obtained For further results see Table 4

b) Gel *precipitation reactions* —Gel precipitation reactions with serum from the animals in this group showed two precipitation lines with normal human serum and one precipitation line with horse serum There was reaction of non identity between these lines (see Fig 6)

Control Experiments

a) *Schultz Dale experiments* —All the antigens used in this experiment were tested on ileum from three normal non immunized guinea pigs In no instance was a positive reaction obtained In this connection it must be stressed that dilutions of serum etc always were prepared at least 24 hours prior to the experiments in order to avoid false positive reactions caused by bradykinin like substances

b) *Gel precipitation reactions* —Serum from three normal non immunized guinea pigs was tested against the different antigens used in the experiment No precipitation reactions were seen

DISCUSSION

All attempts at a demonstration of microorganisms as aetiological factors in G B S have been negative Attempts to demonstrate qualita

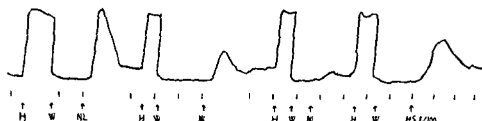


Fig 5
Schultz Dale experiment with ileum from guinea pig sensitized with normal human CSF + diluted normal horse serum H & W see Fig 1 NL = normal human CSF HS = diluted normal horse serum

Immunization with Nervous Tissue

Four guinea-pigs were sensitized with emulsion of nervous tissue from spinal cord, nerve roots and spinal ganglia from a patient with G B S and from a patient without neurological disease

a) *Schultz-Dale experiments* —Using this method no difference was was demonstrable between normal and pathological nervous tissue Normal and pathological CSF gave no reaction with ileum from these animals, indicating that no nervous tissue antigens are present, neither in normal nor in pathological CSF

b) *Gel-precipitation reactions* with serum from these animals were in accordance with the above mentioned results

Immunization with Normal Human CSF + Horse Serum

a) *Schultz-Dale experiments* —Five guinea-pigs were sensitized with a mixture of normal human CSF and normal horse serum diluted to a final concentration of 30 mg of horse protein per 100 ml CSF, upon which segments of ileum from these animals were tested with normal CSF and subsequently with horse serum The purpose of this experiment was to control the negative results in group II and III and to test the sensitivity of the Schultz Dale reaction

TABLE 4
Results of S D Experiments with Ileum from Guinea Pigs Sensitized with Normal Human CSF + Diluted Horse Serum

Primary anaphylactic reaction exhausted with	Secondary anaphylactic reaction with		
	NCSF	NS	HS
Normal CSF (NCSF)			+
Normal human serum (NS)	—		+
Horse serum (HS)	+	+ (4 out of 6)	—

In all the experiments in this group it was possible to elicit an anaphylactic reaction with normal human CSF, a subsequent challenge

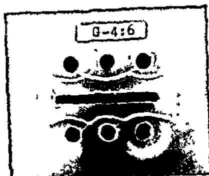


Fig 6

Gel precipitation reaction *Central well* serum from guinea pig sensitized with normal CSF + diluted horse serum *Cups* 1 Normal horse serum 2 Normal human serum 3 Normal human gammaglobulin 4 Normal horse serum 5 Normal human gammaglobulin 6 Normal human serum

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DISCUSSION

All attempts at a demonstration of microorganisms as aetiological factors in G B S have been negative Attempts to demonstrate qualita

tive differences between the CSF protein from normal individuals and from patients with G B S have also been futile In the present investigation it was attempted to reveal such qualitative differences or microbial aetiology using immunological methods To this end experimental animals were immunized with pathological CSF showing the very characteristic elevated protein content Immunological studies could then be performed with serum as well as with isolated organs from these animals It was to be anticipated that a possible pathological antigen would be present in minute amounts and in a mixture containing many other antigens Therefore it was necessary to use a highly sensitive technique providing at the same time a possibility of a qualitative analysis

The *gel-precipitation reaction is known to give an excellent* qualitative analysis, but this method is not very sensitive The Schultz-Dale reaction, however, fulfils admittedly both requirements The extreme sensitivity was confirmed by the authors, showing that even 0 5 μg of antigen, corresponding to 25 nanogram per ml, could elicit a positive reaction The superior sensitivity of this method compared to the gel precipitation *method was demonstrated by the fact, that* positive reactions constantly could be obtained with ileum from sensitized animals if normal CSF were used as antigen, while this antigen in gel-precipitation reactions only occasionally gave a faint precipitation line

To control whether it were possible by this method to distinguish different antigens, small amounts of horse serum were added to normal CSF, and a group of animals were sensitized with this mixture With ileum from these animals it proved possible to exhaust a positive reaction with human protein and still elicit a reaction with horse protein and vice versa The sensitivity to the secondary antigen was somewhat reduced although still superior to that obtained by other methods

Previous investigations (2) have shown, that the blood-liquor barrier in G B S is damaged with subsequent passage of all serum protein fractions into the CSF This was confirmed in our experiments, in which ileum from guinea-pigs sensitized with pathological CSF reacted with human serum After the ileum had been made refractory to human serum no reactions could be elicited with pathological CSF These experiments have shown that it is impossible, even with this very sensitive method, to demonstrate any qualitative differences between the proteins in CSF from patients with G B S and normal human serum proteins The results were supported by the less sensitive gel-precipitation reactions

With ileum from guinea pigs sensitized with pathological CSF, it was repeatedly attempted to elicit a reaction with pathological CSF after the ileum had been rendered refractory against normal CSF This was possible only in two out of 15 experiments, thereby indicating that the proteins in pathological CSF did not differ qualitatively from the proteins in normal CSF Thus the experiments were discordant with the

results obtained by *Clausen, Krogsgård & Quaade* (2) Using immuno-electrophoresis these authors showed, that CSF from patients with G.B.S. in addition to the protein fractions found in normal CSF, contained alpha 2-lipoprotein and alpha-2-macro-globulin. This discrepancy however, can be explained by the superior sensitivity of the S-D reaction revealing small amounts of these protein fractions, also in normal CSF. In the two cases, in which a positive reaction was obtained with pathological CSF after exhaustion of the reaction to normal CSF the difference in total protein content in normal and pathological CSF was very considerable. Control experiments have shown that the refractory state is not absolute but depending upon quantitative factors. If the dose of antigen used to render the ileum refractory is too small in relation to the secondary dose, a positive anaphylactic reaction against the same antigen can be elicited twice.

Guinea pigs were also sensitized with pathological nervous tissue to see whether antigenic substances not found in normal nervous tissue could be demonstrated by this technique. S-D reactions with ileum from these guinea-pigs showed no reactions characteristic of pathological nervous tissue or pathological CSF. Ileum from animals sensitized with pathological CSF gave an anaphylactic reaction with pathological nervous tissue; this reaction, however, could not be elicited after previous treatment with normal human serum or normal human CSF, indicating that the antigen responsible for the reaction was not a tissue antigen peculiar to nervous tissue, but only a part of the serum proteins.

Within the limits of its sensitivity the gel-precipitation method gave results which were in accordance with those obtained with the S-D reaction.

SUMMARY AND CONCLUSION

32 guinea pigs were sensitized with CSF and nervous tissue from normal individuals and patients with Guillain-Barre's Syndrome. Isolated segments of ileum and serum from these animals were used in immunological studies comprising Schultz Dale reactions [illegible]

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IN VITRO ACTIVITY OF PAROMOMYCIN

Spectrum and Sensitivity Test by the Prediffusion Method

By

NIKOLAJ MORTENSEN and AAGE HEIN CHRISTIANSEN

Received 16 1 65

Paromomycinum (WHO, NFN), paromomycin (BAN) or Humatin* (4) is an antibiotic produced by streptomyces from various sources Chemically it consists of four carbohydrate components linked glycosidically

The antibacterial spectrum, the partial cross resistance to streptomycin and complete cross-resistance to neomycin and kanamycin (17) and the regular human cutaneous cross sensitivity between neomycin and paromomycin (19) testify to the close biological kinship between paromomycin and the members of the streptomycin neomycin group The precise nature of its mode of action is not known However, experiments with suspensions of rat liver mitochondria are claimed to demonstrate an inhibition of the oxidative phosphorylation of members of the citric acid cycle (3)

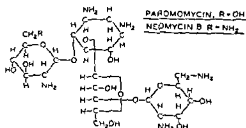


Fig 1
Chemical structure of paromomycin and neomycin B adapted from *Tatsuoka et al* (22)

Paromomycin has been shown to have antimicrobial action *in vitro* against a wide range of gram positive and gram negative bacteria (2 10 15 20) Animal experiments have shown paromomycin to possess marked neural and renal toxic properties after systemic application (4 6) Lower potency in neuromuscular blocking action of paromomycin in comparison with neomycin is claimed (1) Minimal gastrointestinal absorption protects against major toxic action of paromomycin (4)

In humans, the substance has been found to reduce the number of bacteria in the faeces following oral medication (23) Toxic and side effects after oral use include mild to moderately severe diarrhoea (18, 23) Studies on gastro-intestinal drug concentrations are not available

Although preparations for parenteral administration are not generally accessible, serum antibiotic activity after intramuscular injection of 600 mg of paromomycin in four volunteers has been recorded as 10 16, 7 and 9 μg per ml six hours *p i* (7)

Paromomycin has been used in treatment of salmonella, shigella and other enteric infections with results comparable to treatment with similar antibiotics (12, 18, 24)

Development of partial resistance *in vivo* has been observed (5) Staphylococcal enterocolitis has occurred due to coagulase-positive strains resistant to kanamycin, neomycin and paromomycin following *prophylactic preoperative bowel sterilization* (9)

The purpose of the present work is to study the antibacterial spectrum of paromomycin *in vitro* and to elaborate a conversion graph relating bacterial susceptibility and inhibition zones of a paper disc method using 20 hours of prediffusion as employed in the routine sensitivity tests in this laboratory

MATERIAL AND METHODS

Bacteria 129 strains representing common pathogens isolated from clinical specimens and preserved by lyophilization were used to determine inhibitory concentrations (Table 1) 40 of these, showing different degrees of sensitivity, were selected for the agar diffusion tests

Medium Ox heart broth with approximately 1 8 per cent agar, 10 per cent defibrinated horse blood 1 per cent glucose 0 3 per cent sodium chloride 0 2 per cent Na_2HPO_4, 12 H_2O, pH 7 3 7 4

Antibiotic Humatin® sulphate (B No 41941) supplied by Messrs Parke Davis & Company as a white water soluble powder declared to contain 700 mg paromomycin per g For determination of the 50 per cent and the minimum inhibitory concentrations by the plate dilution method plates containing 64 32 16 8 4 2 1 0 5 and 0 μg of Humatin® sulphate per ml were prepared

Discs Sterile filter paper discs (Carl Schleicher & Schull Germany, No 2247) Diameter 6 mm Average absorption power 22 6 mg The discs were made to give an amount of 12 5 25 50 and 100 μg of paromomycin sulphate per disc Uniformity range (16) 2 5 mm (n = 36)

Inoculation A 20 hour culture in serum broth (8 hours for *Diplococcus pneumo*

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hours at room temperature (22) Immediately following removal of the [illegible]ses duplicate plates were inoculated with 0 1 ml of the serum broth cultures diluted as

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The growth was graded

nt inhibitory concentra

tions The lowest concentration of [illegible] sulphate in the plate without visible growth was taken as the minimum inhibitory concentration

Zones of inhibition were measured to the nearest 0 5 mm

RFSUITS

The 50 per cent and the minimum inhibitory concentrations of the strains examined are listed in Table 1

TABLF 1

50 per cent and Minimum Inhibitory Concentrations of Paromomycin Sulphate against Different Bacterial Strains

	Number of strains	50 per cent inhib conc (g ml)	Min Inhib conc (g/ml)
Escherichia coli	8	0 8 5 7	2 16
Klebsiella	7	0 5 1 2	1 2
Salmonella	8	0 7 4	1 8
Shigella	8	0 7 5 7	2 16
Proteus			
swarming	6	2 8 9 5	8 16
non swarming	22	1 4 45	2 >64
Pasteurella pseudotub	8	1 4 4 8	2 8
Pseudomonas	8	27 >90	64 >64
Listeria	8	<0 5 1 2	<0 5 8
Staphylococcus			
coagulase neg	8	<0 5 2 8	<0 5 8
coagulase pos	8	<0 5 0 7	<0 5 2
Streptococcus			
enterococcus	8	>90	>64
other	7	32 >90	64 >64
Pneumococcus	7	2 8 8	8 16
Bact anitratum	8	0 6–2 4	1 8

A conversion graph showing the relation between the 50 per cent inhibitory concentration and the diameter of the inhibition zone for discs containing 50 and 100 µg drawn as lines is shown in Fig 2 The points indicate the average of double determinations of one strain with the 50 µg disc

DISCUSSION AND CONCLUSIONS

The inhibitory concentrations confirm previous reports of antibacterial activity of paromomycin and correspond well with the values of minimum inhibitory concentrations published by other authors (2 10 15 20) as regards susceptibility of different genera Minor differences may be attributable to differences in species of organisms and in the technique used Staphylococci and enterobacteria are generally less resistant than pseudomonads and str ptococci ...

paper diffusion technique (8 25) ...ations (14) a single disc method allows quantitative readings to be made The size of inhibition zones agrees well with the inhibitory concentration values and this result also con

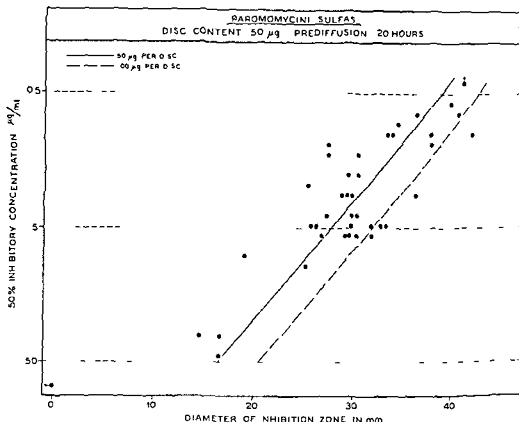


Fig 2

Results of plate dilution and prediffusion tests Points indicate average of double determinations with a disc containing 50 µg of paromomycin sulphate Horizontal lines mark limits between sensitivity groups

firms that application of a 20 hour-prediffusion period is practicable For the special advantages of the long prediffusion period see (22).

The difference between the 50 per cent and minimum inhibitory concentrations is quite constant for most of the strains examined and does not exceed 1 or 2 two-fold dilution steps Only in the case of Listeria was a difference of 1 to 4 steps found This is taken to reflect a greater intrinsic inhomogeneity or "tailing" (13) of these strains when tested with paromomycin

For "tailing" strains, calculation of the 50 per cent inhibitory concentration by the Karber method improves the correlation between the minimum inhibitory concentration and the diameter of the inhibition zone With these strains conversion of the diameter of the inhibition zone by means of a standard graph into 50 per cent inhibitory concentrations will indicate a relatively greater sensitivity than by relating inhibition zones to minimum inhibitory concentrations

Grouping of bacteria according to their sensitivity is usually done by taking into account clinical experience and obtainable serum drug levels Comparison of clinical reports of cases treated with paromomycin with records of the sensitivity of the pathogenic strains could facili-

tate this grouping but has not been published In the case of paromomycin where application is only oral and intestinal absorption negligible arbitrary methods of grouping are necessary

Adoption of the same criteria as with *framycetin (11) results in the* grouping shown in Table 2

TABLE 2

Grouping of Bacterial Strains Based on 50 per cent Inhibitory Concentrations and Corresponding Diameters of Inhibition Zones Using Discs Containing 50 µg of Paromomycin Sulphate

	Diameter of inhib zone in mm	50 per cent inhib conc µg ml	Sensitivity group
Sensitive	≧ 38	≦ 0 5	+ + +
Moderately sensitive	< 38 ≧ 27	> 0 5 ≦ 5	+ +
Relatively resistant	< 27 ≧ 16	> 5 ≦ 50	+
Resistant	< 16	> 50	0

SUMMARY

Reports on the antibacterial spectrum of paromomycin are confirmed by determination of the 50 per cent and the minimum inhibitory concentrations of 129 strains representing common pathogens

A conversion graph is made between the diameter of inhibition zone in the paper disc diffusion method using 20 hour *prediffusion and* 50 per cent inhibitory concentrations in plate dilution experiments

Results of determinations are classified empirically in sensitivity groups

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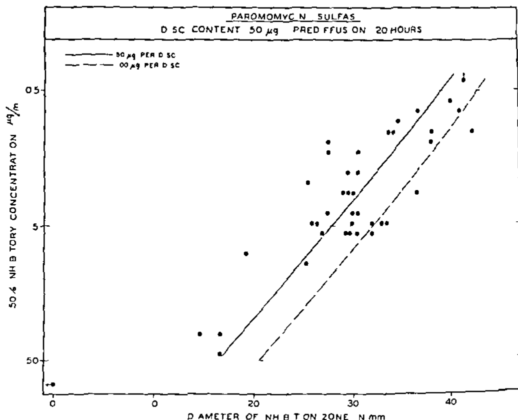


Fig 9

Results of plate dilution and prediffusion tests Points indicate average of double determinations with a disc containing 50 µg of paromomycin sulphate Horizontal lines mark limits between sensitivity groups

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The Virus Department B National Institute of Public Health
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University of Oslo Norway (Head J Jonsen, M D)

INVESTIGATIONS OF SOME EFFECTS OF HUMAN SALIVA ON INFLUENZA VIRUS

3 *Virus Antibodies in Saliva*

By

GUNNAR ROLLA

Received 16 1 65

Many infective agents enter the human body through the mouth The presence of antibodies in saliva may therefore have relation to the pathogenicity of a number of bacteria and viruses

The presence of bacterial agglutinins in saliva is well established (9, 12), and precipitation and complement fixation of bacterial antigens by saliva have been demonstrated (4, 13)

The presence of gamma-globulin in saliva was shown by several workers (5, 6, 7), using gel precipitation and immune-electrophoresis techniques

The following conclusions were drawn from earlier investigations

1) In man, monkey, rabbit, and rat acquired antibodies against bacterial antigens may be found in saliva when they are present to a high titre in serum
2) There is no fixed relationship between the titres in serum and saliva

Publications concerning virus antibodies in saliva are few only *Jungeblut et al* (2) have reported haemagglutination inhibition of Columbia SK virus by the saliva from patients suffering from paralytic polio Some of these patients are known to have serum antibodies against Columbia SK virus (3)

Marmion et al (8) found a neutralizing factor against influenza virus in the expectorate of patients suffering from chronic bronchitis They stated that the identity of this factor was undecided

The experiments described in this paper intend to estimate the amount of antibody secreted in saliva after injections of influenza virus antigens [illegible]

we [illegible]

[illegible] in saliva after immunization will be of the utmost importance in the present investigation

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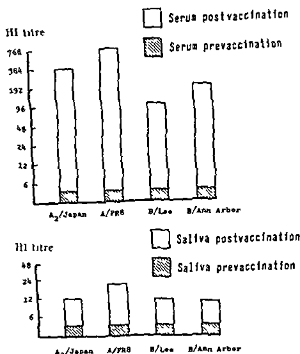


Fig 1

Change in haemagglutination inhibition titre in serum and saliva after vaccination with influenza virus antigens

TABLE 2

HI Tests of the Pooled Saliva Samples before and after Injections of Influenza Virus Antigens

Virus antigen	Prevaccination samples		Postvaccination samples	
	Before RDE	After RDE	Before RDE	After RDE
A_2/Japan 305/57	80	<6	80	12
A/PR 8	1280	<6	1280	24
B/Lee	40	<6	80	12
B/Ann Arbor 1/59	40	<6	80	12

2) Comparison of the HI Titres of Parotid and Sublingual Saliva after *Injection of Influenza Virus Antigens*

Experiments described in a previous paper (10) have shown that sublingual saliva has a higher activity than parotid saliva when tested by HI tests. A comparison of pooled postvaccination sublingual and parotid saliva was therefore thought to be of interest. The saliva samples were treated with cholera filtrate as described earlier. The results are given in Table 3.

MATERIAL AND METHODS

4 subjects were tested, two of each sex, aged 21–37 years They were in good health, had not been ill and did not receive any medication during the three months preceding the experiment As *antigens* served commercial influenza vaccine (SIFF)[1] Two injections of 1 ml were given at an interval of one week to each subject The vaccine contained A_2 and B antigens

Saliva Unstimulated saliva was collected directly from the mouth Sublingual saliva was collected from the sublingual area using pipettes while the parotid ducts were blocked with cotton rolls Parotid saliva was squeezed out of the cotton rolls blocking the parotid ducts To the samples were added antibiotics (10), and they were stored frozen at —20° C Before titrations, the samples were thawn and centrifuged in a refrigerated centrifuge (5000 r p m for 15 minutes) The supernatants were used Saliva and serum samples were collected and pooled before the first injection of antigen and three weeks after the second injection

The saliva samples were treated with cholera filtrate to remove unspecific inhibitors One volume of saliva was mixed with five volumes of cholera filtrate (N V Philips Duphar) and the mixture incubated over night at 37° C followed by inactivation for one hour at 56° C Penicillin and streptomycin were added to the cholera filtrate The samples were then absorbed with 10 per cent red cells for 30 minutes at 4° C, and then centrifuged to remove agglutinins adsorbed to the red cells

Haemagglutination inhibition tests were performed as described in a previous paper (10)

Virus The strains employed are given in the tables

RESULTS

1) *Comparison of Haemagglutination Inhibition Titres of Serum and Saliva before and after Injection of Virus Antigens*

The pooled sera and saliva samples collected before and after the vaccination with influenza virus antigens were compared HI-tests were performed on the samples before and after treatment with cholera filtrate The results are given in Tables 1 and 2 (Fig 1)

TABLE 1

HI Tests of the Pooled Sera before and after Injections of Influenza Virus Antigens

Virus antigen	Prevaccination samples		Postvaccination samples	
	Before RDI	After RDI	Before RDI	After RDE
A_2/Japan 305/57	160	<6	768	384
A/PR 8	160	<6	768	768
B/Lee	80	<6	384	96
B/Ann Arbor 1/59	80	<6	384	192

It is seen from the tables that prevaccination sera and saliva samples treated with cholera-filtrate showed no (<6) HI-activity

The corresponding postvaccination tests showed a good increase of the HI-titre of the sera and a moderate increase of the saliva samples

1 National Institute of Public Health Oslo Norway

DISCUSSION

The results in Table 1 show a rise of HI activity in the pooled post vaccination sera It is also seen that the treatment with cholera filtrate destroys all unspecific haemagglutination inhibition in serum Table 2 shows that the treatment with cholera filtrate destroys the inhibitory activity in pre vaccination saliva This indicates that the activity found in post vaccination saliva was caused by the injected influenza virus antigens

It has been shown in a previous publication that the content of haemagglutination inhibitors are usually higher in sublingual than in parotid saliva (10) From Table 3 it may be seen that post vaccination samples of sublingual and parotid saliva contain equal amounts of inhibitor This result also supports the view that the activity in post vaccination saliva is of antibody character

The inhibition of the haemagglutinins of A/PR 8 was obtained with a saliva dilution of 1/24 while the other viruses were inhibited by a dilution of 1/12 In post vaccination serum A/PR 8 was also inhibited to a higher titre than the other viruses tested

No change in HI activity takes place during incubation with A/PR 8 or B/Lee (Table 4) Unspecific haemagglutination inhibition is destroyed by this treatment (11)

The experiments described show an increase in virus antibodies in saliva after immunization with virus antigens

SUMMARY

1 Pooled saliva samples from 4 subjects show increase in haemagglutination inhibition titre after vaccination with influenza virus antigens

2 Evidence of the antibody character of the increased haemagglutination inhibition activity in post vaccination saliva is presented

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8 *Harm[illegible]*

TABLE 3

HI-Tests Showing Virus Antibodies in Sublingual and Parotid Saliva after Injection of Virus Antigen*

HI titres

Virus antigen	Parotid saliva		Sublingual saliva	
	Before vaccination	After vaccination	Before vaccination	After vaccination
A_2/Japan 305/57	<6	12	<6	12
A/PR 8	<6	24	<6	24
B/Lee	<6	12	<6	12
B/Ann Arbor 1/59	<6	12	<6	12

* Saliva treated with cholerafiltrate and heated to 56° C

The table shows that there is no difference in the post-vaccination HI-titres of parotid and sublingual saliva These results are in sharp contrast to those obtained with pre-vaccination untreated saliva (10)

3) *Investigation on the Kinetics of the HI-Activity Present in Post Vaccination Saliva during Incubation with Virus*

It has been shown that a marked difference exists between the kinetics of the HI-process of immune antibody and that of the haem agglutination inhibitor of untreated saliva (11) No change of activity is observed during incubation of immune-serum and virus The saliva inhibitor, on the other hand, is destroyed in 2–3 hours, presumably by the neuraminidase activity of influenza viruses This difference may give an indication as to the nature of the HI-activity present in post vaccination saliva treated with cholera-filtrate The results are given in Table 4

TABLE 4

Constancy of HI Titres of Postvaccination Saliva upon Incubation with Influenza Virus*

Time of incubation	A PR 8	B Lee
To	24	12
+ 5 minutes	24	12
+ 10 minutes	24	12
+ 1 hour	24	12
+ 2 hours	24	12
+ 3 hours	24	12

* Treated with cholerafiltrate and heated to 56° C

Procedure To 0 25 ml of saliva or saliva dilutions were added 0 25 ml of allantoic fluid containing 4 ADS of virus After incubation for the stated times 0 25 ml of a suspension of chick red cells were added

No change in HI-titre was demonstrable when A PR 8 and B Lee were incubated with post vaccination saliva, which had been treated with cholera-filtrate

DISCUSSION

The results in Table 1 show a rise of HI activity in the pooled post vaccination sera It is also seen that the treatment with cholera filtrate destroys all unspecific haemagglutination inhibition in serum Table 2 shows that the treatment with cholera filtrate destroys the inhibitory activity in pre vaccination saliva This indicates that the activity found in post vaccination saliva was caused by the injected influenza virus antigens

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IRREVERSIBLE DETOXIFICATION OF PURIFIED DIPHTHERIA TOXIN

By

I SCHEIBEL and P ELO CHRISTENSEN

Received 26 ii 65

Until recently the interaction between diphtheria toxin and formaldehyde, resulting in a non toxic modification (toxoid) with retained antigenic capacity, has been considered irreversible This is, of course, an ultimate requirement for vaccine preparation

In order to reduce the frequency and severity of complications following diphtheria vaccination, producers have made efforts to purify the antigenic material as much as possible Purification procedures applied to crude or ultrafiltrated toxoids yielded stable products The purification obtained in this way was not always satisfactory and it appeared that better results could be obtained by purification of the toxin before detoxification

However, *Linggood, Stevens, Fullhorpe, Woiwod & Pope* (2) showed that attempts to detoxify highly purified diphtheria toxin by means of formalin alone resulted in a product which, after dilution and storage at room temperature or 36° C, gradually became toxic A similar observation was made in 1937 by *Wadsworth, Quigley & Sickles* (8) with partly purified diphtheria toxin The toxicity of the reversed product was low compared to that of the original toxin but sufficient to disqualify it for immunization of humans

Linggood et al (2) succeeded in inhibiting this reversal by the addition of certain amino acids during the detoxification process

The purpose of the present work was to investigate the behaviour in this respect of the purified diphtheria toxins prepared in this laboratory

MATERIALS AND METHODS

Diphtheria toxin was prepared from shake cultures of *C diphtheriae* Park Williams No 8 (substrain CN 2000) in tryptic digest medium (modification of the medium described by *Pope & Linggood* (4)) The crude culture filtrates ranging from 140 160 Lf per ml were concentrated by ultrafiltration and purified by the ammonium sulphate-charcoal method described by *Pope & Stevens* (5) This resulted in products containing 2000–2200 Lf units per mg of trichloracetic acid precipitable nitrogen and about 10 per cent less per mg of total nitrogen

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Our thanks are due to Miss *F Steenberg* for excellent technical assistance

Toxoiding was carried out at 500 or 1000 Lf per ml in saline containing 10 mg per cent thiomersal

Phosphate buffer was used to stabilize the pH which was finally adjusted to the desired values

Formaldehyde was added as formalin solution (36 per cent w/v)

The amino acids investigated l lysine (Merck), glycine (Merck) and dl alanine (NBC) were added as 10 per cent solutions

The preparation was made aseptically from sterile components without any final sterile filtration

The mixture was kept at 32° C for four weeks

Toxicity testing after toxoiding was carried out by intracutaneous injection of 50 or 100 *Lf* into rabbit or guinea pig No reaction to this *i e* less than 0 02 or 0 01 minimal skin reacting doses (DRM) per Lf respectively, is taken as indication of non toxicity

Reversal tests were carried out by a method almost similar to that described by *Linggood et al* (2) After removal of HCHO and amino acids by dialysis dilutions to approximately 60 Lf per ml of the various detoxified toxins were made in sterile phosphate buffered saline pH 7 3 containing 10 mg per cent thiomersal These were kept at *32° C* Samples were taken after 4 and 8 weeks and tested for toxicity by intracutaneous injection into rabbits and guinea pigs of 0 1-0 2 ml representing 5 to 10 Lf Thus no reaction means less than about 0 1-0 2 DRM per Lf and is taken as indication of stability smaller concentrations of toxin not being measurable under the given conditions Whenever quantitative estimations were carried out tenfold dilutions were used

If reversal occurred this generally took place already after four weeks It might sometimes require up to eight weeks but has not been seen to occur after that *time*

Antigenic potency Varying dilutions in saline of the toxoid to be tested were given subcutaneously in doses of 0 5 ml to groups of 15 guinea pigs weighing 260–280 g

The toxoids were tested both after adsorption to $Al(OH)_3$ gel (1 mg Al per ml toxoid dilution of 50 flocculation units) and in the plain state

Blood sampling was carried out by heart puncture four weeks after the injection *In a few cases where plain toxoids were concerned the injection was repeated* after four weeks and blood samples were taken again two weeks after the last injection

The responses were estimated by titrating the antitoxin in the individual sera by the haemagglutination method (7) in relation to the international titres for in vivo titration of diphtheria antitoxin and expressed in international units (6) The logarithmic mean response was calculated for each group

RESULTS

In the first experiment, a purified toxin *from the routine production* was detoxified under conditions comparable to those used by the British workers as regards concentrations of amino acids and formaldehyde The toxin concentration was about 500 Lf per ml Results with concentrations of 1000 Lf per ml did not differ essentially from those with *500 Lf per ml*, and are therefore not presented here Samples of the toxin were detoxified in 0 025 M phosphate buffer with formaldehyde 0 06 M alone or together with alanine 0 1 M, glycine 0 1 M, lysine 0 025 M and alanine *0 075 M + lysine 0 025 M The initial pH* was *adjusted* to 7 0 During the process it dropped to values between 6 5 and 6 8 for the samples containing amino acid, and remained unchanged in the control

After storage for four weeks at 32°, all the samples were non toxic, *i e* contained less than 0 02 DRM per Lf No measurable decrease in the flocculating values had occurred during toxoiding in any of the samples

The results of the reversal testing are given in Table 1. The sample detoxified with formalin alone increased its toxicity more than 1500 times and the alanine and glycine toxoided samples more than 50 times, whereas the samples toxoided with lysine and alanine + lysine did not show any detectable increase in toxicity.

TABLE 1

Stability at 3[illegible]° of non-toxicity and Flocculation Titres (Lf) of Purified Diphtheria Toxin Detoxified with Formaldehyde 0.0[illegible] M at Lf 500 per ml in the Presence of and without Amino Acids

Reversal testing after 8 weeks at 32°	Alanine 0.1 M	Glycine 0.1 M	Lysine 0.025 M	Alanine 0.075 M + Lysine 0.025 M	No amino acid
DRM per Lf	1	1	< 0.1*	< 0.1*	30
Lf decrease in %	11	20	10	10	12

* Smallest measurable toxin concentration. No reaction to this concentration is considered as indicating stability.

The loss in flocculating activity during 8 weeks of reversal testing was 20 per cent for the glycine samples and about 10 per cent for the other samples. This is somewhat below the values found by *Linggood et al.* (2), probably because the temperature used by these authors was 36° as against 32° in this study.

Thus we were able to reproduce the results obtained by *Linggood et al.* (2) in the following respects: 1) Purified diphtheria toxin detoxified with formalin alone reverted to toxicity on dilution. 2) Irreversible detoxification could be obtained in the presence either of lysine 0.025 M or of lysine 0.025 M + alanine 0.075 M.

Contrary to *Linggood et al.* (2) it was not possible to obtain completely stable toxoids with alanine or glycine *under the conditions used*. However, the increase in toxicity for the alanine and glycine toxoids was only moderate as compared to the toxoid without amino acids.

From a concentration of 50 Lf per ml all the toxoids could be totally adsorbed by a concentration of aluminium hydroxide gel corresponding to 1 mg of Al per ml in the *adsorption mixture*.

The antigenicity of the different toxoids will be discussed in a subsequent section.

Toxoiding of successive preparations of purified toxin from the routine production, each representing 2 300 litres of crude toxin, with formaldehyde and lysine under the same conditions as described above disclosed that although the purities of the toxins were practically uniform (approximately 2000 Lf per mg of nitrogen), stable toxoids were not obtained regularly.

To elucidate the reason for this, a series of experiments was carried

TABLE 2

Stability at 32° of non toxicity and Flocculation Titres (Lf) of Purified Diphtheria Toxin Detoxified at Lf 1000 with Varying Ratios of Formaldehyde and Lysine and without Lysine at Varying pH

Detoxification conditions	Group 1					Group 2					Group 3				
Phosphate buffer			0 1 M					0 1 M					0 1 M		
Lysine			0 06					0 025					0 01		
HCHO			0 06					0 06					0 06		
HCHO/Lysine			1					2 4					6		
Initial pH	6 4	6 7	7 0	7 3	7 6	6 4	6 7	7 0	7 3	7 6	6 4	6 7	7 0	7 3	7 6
After 4 weeks at 32°															
pH	6 3	6 6	6 8	7 0	7 3	6 4	6 6	6 8	7 1	7 3	6 4	6 7	6 9	7 2	7 4
Lf decrease in %	0	0	10	0	0	0	0	0	0	0	15	0	0	0	0
Reversal testing															
Skin reaction* to 7 Lf	0	0	0	0	0	0	0	0	0	0	++	+	0	0	0
Lf increase in %	0	0	0	12	0	0	0	12	0	0	12	0	0	12	0

TABLE 2 (cont)

Stability at 32° of non toxicity and Flocculation Titres (Lf) of Purified Diphtheria Toxin Detoxified at Lf 1000 with Varying Ratios of Formaldehyde and Lysine and without Lysine at Varying pH

Detoxification conditions	Group 4					Group 5					Group 6				
Phosphate buffer			0 1 M					0 025 M					0 1 M		
Lysine			0 0125 ,,					0 025 ,,					0 ,,		
HCHO			0 03 ,,					0 06					0 06 ,,		
HCHO Lysine			2 4					2 4							
Initial pH	6 4	6 7	7 0	7 3	7 6	6 4	6 7	7 0	7 3	7 6	6 4	6 7	7 0	7 3	7 6
After 4 weeks at 32° pH	6 4	6 7	6 9	7 2	7 4	6 2	6 4	6 6	6 7	6 9	6 4	6 7	7 0	7 3	7 5
Lf decrease in %	15	11	0	0	0	0	0	0	0	0	12	0	0	11	0
Reversal testing Skin reaction* to 7 Lf	++++	+++	++	+	0	++++	++	+	0	0	++++	+++	++	++	+
Lf decrease in %	0	0	12	0	10	0	0	0	0	10	0	0	0	12	0

* 0 = no reaction + = ≤ 10 mm of redness ++ = 10 18 mm of redness
+++ = 15 20 mm of redness and induration ++++ = 20 25 mm of redness and induration

TABLE 2

Stability at 32° of non toxicity and Flocculation Titres (Lf) of Purified Diphtheria Toxin Detoxified at Lf 1000 with Varying Ratios of Formaldehyde and Lysine and without Lysine at Varying pH

Detoxification conditions	Group 1					Group 2					Group 3				
Phosphate buffer			0 1 M					0 1 M					0 1 M		
Lysine			0 06					0 025 ,,					0 01 ,		
HCHO			0 06 ,					0 06					0 06 ,,		
HCHO/Lysine			1					2 4					6		
Initial pH	6 4	6 7	7 0	7 3	7 6	6 4	6 7	7 0	7 3	7 6	6 4	6 7	7 0	7 3	7 6
After 4 weeks at 32°															
pH	6 3	6 6	6 8	7 0	7 3	6 4	6 6	6 8	7 1	7 3	6 4	6 7	6 9	7 2	7 4
Lf decrease in %	0	0	10	0	0	0	0	0	0	0	15	0	0	0	0
Reversal testing															
Skin reaction* to 7 Lf	0	0	0	0	0	0	0	0	0	0	++	+	0	0	0
Lf decrease in %	0	0	0	12	0	0	0	12	0	0	12	0	0	12	0

indicates that the ionic strength, or perhaps the phosphate ions as such, may also be of significance for the course of the reaction

Without the addition of lysine the process reverts over the whole range of pH (group 6)

It is seen from group 3 to 6 that the degree of reversal increases with decreasing pH

The decrease in flocculating value of the different samples during toxoiding and reversal testing can be seen from the last lines in the middle and lowermost part of the table Where diminished values were observed, these were not systematic and of doubtful significance

As regards the flocculation time, the maximal increase observed during toxoiding was about 3 times, this occurring at the two highest pH values in group 2, 3 and 4 only Kf was unchanged at pH 6 4 in groups 3, 5 and 6, and was increased about twice in the rest of the samples

In order to elucidate the influence of the time relationship between the addition of HCHO and lysine and the stability of the reaction product an experiment was set up in which lysine was added at varying intervals after the addition of formaldehyde and reversal testing carried out 7 to 28 days after the addition of lysine The design and results of this experiment are shown in Table 3

TABLF 3

Influence of Time of Contact of Purified Diphtheria Toxin (1000 Lf per ml) Formaldehyde (0 06 M) and Lysine (0 025 M) at pH 7 5 (Phosphate Buffer 0 1 M) on the Stability of the Reaction

Days at 37° with HCHO alone	DRM per Lf on addition of lysine	Skin reaction* to 7 Lf on reversal testing		
		No lysine	Days after addition of lysine 7	28
0	4 × 10		20 RI	0
4	<1 × 10-2		18	0
7		30 RIN	15	0
14			5	0
21			0	0
28	,	20 RI	0 ,	0

* Figures = size of reaction in mm R = redness I = induration N = necrosis 0 = no reaction

It is evident that lysine may be incorporated into the molecule and may counteract the reversal to toxicity, even though the HCHO-protein reaction has proceeded to the end, as estimated from the non-toxicity of the complex

If lysine is present in the HCHO-toxin mixture for one week only, the stability of the toxoid depends upon the preceding time of contact between the toxin and HCHO The stability increases with the duration of this contact and is total after three weeks of HCHO toxin contact If lysine is present for four weeks in the HCHO toxin solution, stable

out with varying concentrations of lysine and formaldehyde in 0 025 M phosphate buffer Lysine was used in three different concentrations, viz 0 025 M, 0 05 M and 0 1 M, and formaldehyde in concentrations of 0 03 M, 0 06 M and 0 09 M The molar concentration ratio HCHO lysine was varied between 0 6 and 2 4 The initial pH was adjusted to 7 0 in most of the samples and in a few cases to 7 5 or 8 0 However, at the buffer strength used (0 025 M) the pH dropped markedly during the detoxification period, as mentioned previously

The results of these experiments were rather confusing and seemed to be mutually conflicting From the sum of them, it became evident *that the reason may be that the interrelationship of the factors examined, rather than any single factor, was responsible for the course of reaction*

This presumption is supported by the results of the experiment shown in Table 2, where five pH values ranging between 6 4 and 7 6 were used for each of six groups of test samples varying as regards the relative and actual concentrations of lysine and formaldehyde The composition of the groups and the results are given in Table 2

In order to keep the pH fairly constant during the whole process, the detoxification was carried out in 0 1 M phosphate buffer, except for group 5 where the previously used strength of 0 025 M was used for comparison

All the mixtures, except two, proved non-toxic already after one week *of toxoiding These were the two samples with the lowest pH values* in the group with 0 03 M HCHO (group 4), which contained 5 and 0 1 DRM per Lf, respectively

Reversal testing after one week of detoxification showed that the reaction was reversible in all samples, and that the two groups 5 and 6 showed a reversal degree at least ten times higher than that of the other groups (The "one week" results are not included in the table)

After four weeks at 32°, all samples were found to be non-toxic

The lowermost part of Table 2 presents the results of the reversal tests on these samples The essential points can be summed up as *follows* —

Stable toxoids were obtained through the whole range of pH between 6 4 and 7 6 by the use of formaldehyde 0 06 M lysine 0 025 M or more, and phosphate buffer 0 1 M (group 1, 2), whereas at a lysine concentration of 0 01 M stable detoxification only took place at pH 7 0 or above (group 3) If a HCHO/lysine ratio of 2 4 were maintained as in group 2 but the actual concentrations were halved a stable toxoid was obtained only at pH 7 6 (group 4)

With the same conditions as in group 2 except that the buffer concentration was four times lower, an initial pH of at least 7 3 was required for irreversible toxoiding (group 5) The fact that the higher ion strength in group 2 resulted in an irreversible reaction, at the same pH values as the final pH of two of the reverting samples in group 5,

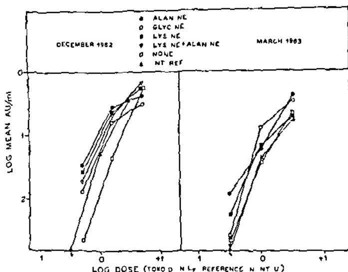


Fig 1

Dose response curves for $Al(OH)_3$ adsorbed diphtheria toxoid detoxified from purified toxin in the presence of and without amino acids An arrow attached to a point indicates that some of the animals in the group did not give a measurable response

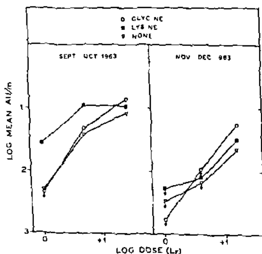


Fig 2

toxoids are obtained, irrespective of previous contact between toxin and formaldehyde

In accordance with these observations, routine preparations of reverting toxoid detoxified from purified toxins without the presence of lysine, or with lysine under unfavourable conditions, were made stable by renewed treatment with formaldehyde and lysine At a toxoid concentration of approximately 2000 Lf in 0 025 M phosphate buffer at pH 7, this was obtained by treatment for 2 weeks at 32° with 0 06 M HCHO and 0 025 lysine without loss of flocculating activity and with only a slight increase in flocculating time

0 018 M formaldehyde and 0 01 M lysine were likewise tried, and provided that either contact for 4 weeks or a pH of about 7 5 were used this also induced irreversibility

Linggood et al (2) observed that the incorporation of different amino acids into the toxoid molecule resulted in pronounced differences in the antigenic potency for guinea pigs They found that, compared to toxoid detoxified without amino acids, the potency of toxoid detoxified with glycine was reduced very markedly, while that of toxoid detoxified with lysine was increased

Their experiments were carried out with plain toxoids only, and with a one-dosage method, namely two injections of one Lf given at an interval of 4 weeks

By determining the dose response curve to one injection of the different toxoids, we hoped to obtain more detailed information on this very interesting point

Such experiments were undertaken on the toxoids mentioned in Table 1, both after adsorption to $Al(OH)_3$ and in the plain state

Two assays were carried out with the adsorbed toxoids and the results are given in Fig 1

In the first of these (December 1962) three groups of guinea pigs received 0 5, 1 65 and 5 Lf units, respectively

No significant variation was found between the four amino acid detoxified toxoids, whereas the toxoid detoxified without amino acid elicited significantly lower responses to the smaller doses but not to the highest dose

Except for the lysine alanine toxoid, the experiment was repeated using somewhat smaller doses, *viz* 0 3, 1 0 and 3 0 Lf units The graphical evaluation disclosed only chance variations between the toxoids[1]

It must be concluded, therefore, that incorporation of the different amino acids did not influence the antigenicity of the adsorbed toxoids

Similar determinations were made with the plain toxoids detoxified by the addition of lysine and glycine, and without amino acid Again, two experiments were run, one in September October the second in November December 1963 The results are seen from Fig 2

1 Thanks are due to Mr *M Weis Bentzon* Head of the Statistical Department for carrying out the statistical evaluation of the antigenic potency experiments

amino acid is imperative In this latter process a change of 0 3 in pH may in certain circumstances be decisive for an irreversible course

The discrepancies between some of the observations by the English authors and ours e g as regards the stabilizing effect of alanine and glycine are therefore understandable because of the somewhat different experimental conditions used The same applies to the variation in stability seen by us between different batches of lysine toxoids prepared under conditions believed to be identical

The complexity and the lack of exact knowledge of the underlying chemical reactions make it difficult to arrive at a dependable standard method by which stable toxoids may be obtainable by detoxification of purified diphtheria toxins As a general rule it may be stated that with increasing pH values within the range used the relative and actual concentrations of the other factors examined become less significant

It is interesting that the amino acid may be incorporated successfully at any time during the HCHO protein reaction even after this has proceeded to complete transformation to the non toxic form This indicates that the toxic groups in the molecules need not participate in the reactions responsible for the irreversibility

Nevertheless there is some time relationship between the two reactions since the longer the previous contact of HCHO protein the shorter the time needed for the lysine HCHO protein reaction to become irreversible

We have not been able to demonstrate the improvement in antigenic potency for guinea pigs observed by *Linggood et al* by incorporation of lysine into the toxoid With the lysine toxoid prepared by us the dose response curves both for $Al(OH)_3$ adsorbed and plain toxoid did not vary significantly from those for the corresponding control toxoid The same applied to the response to two injections of 1 Lf

Neither have we been able to confirm the very pronounced decrease in antigenicity by incorporation of glycine reported by the above mentioned authors The dose response curves both for $Al(OH)_3$ adsorbed and plain toxoids did not disclose significant dissimilarities between the glycine toxoid and the corresponding control toxoid although the mean responses to the very low doses had a trend towards lower values for the glycine toxoid Our results thus seem to be more in accordance with those of *Neumuller* (3) who reported an excellent antigenic effect of glycine toxoid

However when carrying out an assay by the two dose method used by *Linggood et al* (2) some decrease in antigenic effect was disclosed the mean of the antitoxic response being significantly lower for the glycine toxoid than for the lysine and control toxoids The change of the molecule can scarcely be very extensive since it does not manifest itself significantly in the dose response curve of single doses

It remains to be shown whether the antigenicity is affected by the conditions used for the amino acid incorporation If so that may be

In the first of these, the toxoids were given in doses of 1, 5 and 25 Lf. The glycine toxoid and the control toxoid behaved alike. The lysine toxoid elicited a significantly better antitoxin response than the two others, except for the highest dose which may have fallen on the asymptotic part of the curve.

When repeated using closer dose intervals (1, 4, 16 Lf units) this difference did not manifest itself significantly. However, numerically the lysine group given the smallest dose again had a higher mean than the two other groups, which, as in the first assay, did not show more than chance variation.

Next, antigenic potency determinations were carried out according to the method used by *Linggood et al.* (2) by giving the 1 Lf group from the last assay an additional injection of 1 Lf 4 weeks after the first one and titrating the response in blood samples taken 10 days later. The results are seen in Table 4.

TABLE 4

Mean Antitoxin Response (log AU/ml) in Guinea Pigs to two Injections of 1 Lf of Plain Purified Diphtheria Toxoid Detoxified with and without Addition of Amino Acids

Amino acid	Nov–Dec 1963	Feb–Mar 1964
Glycine	<−2.27	−1.58
Lysine	−0.38	−0.34
None	−0.80	

Administered in this way, the lysine toxoid and the non amino acid treated toxoid again did not differ more than what may be due to chance, whereas antigenic effect of the glycine toxoid was significantly lower. Only 3 out of 13[1] animals in the glycine group gave a typical secondary response, as compared to 13 out of 14[1] and 15 out of 15 in the two other groups.

On repeating the experiment with the glycine and lysine toxoids, a significantly reduced antigenic effect of the glycine toxoid could again be demonstrated, as shown in the last column of the table.

DISCUSSION

As pointed out by *French & Edsall* (1), the course of reaction between formaldehyde and proteins is very sensitive to conditions. The results of the present study have confirmed this as regards the reaction between formaldehyde and purified diphtheria toxin protein. However, the reaction which transforms the toxic molecule to its non toxic modification seems to be less sensitive to variations in conditions than that which gives an irreversible toxoid and for which the presence of an

1 Reduced numbers due to intercurrent death.

amino acid is imperative In this latter process, a change of 0 3 in pH may, in certain circumstances, be decisive for an irreversible course

The discrepancies between some of the observations by the English authors and ours, *e g* as regards the stabilizing effect of alanine and glycine, are therefore understandable because of the somewhat different experimental conditions used The same applies to the variation in stability seen by us between different batches of lysine toxoids prepared under conditions believed to be identical

The complexity and the lack of exact knowledge of the underlying chemical reactions make it difficult to arrive at a dependable standard method by which stable toxoids may be obtainable by detoxification of purified diphtheria toxins As a general rule, it may be stated that with increasing pH values within the range used, the relative and actual concentrations of the other factors examined become less significant

It is interesting that the amino acid may be incorporated successfully at any time during the HCHO-protein reaction, even after this has proceeded to complete transformation to the non-toxic form. This indicates that the toxic groups in the molecules need not participate in the reactions responsible for the irreversibility

Nevertheless, there is some time relationship between the two reactions, since the longer the previous contact of HCHO-protein, the shorter the time needed for the lysine HCHO-protein reaction to become irreversible

We have not been able to demonstrate the improvement in antigenic potency for guinea pigs observed by *Linggood et al* by incorporation of lysine into the toxoid With the lysine toxoid prepared by us, the dose-response curves both for $Al(OH)_3$-adsorbed and plain toxoid did not vary significantly from those for the corresponding control toxoid The same applied to the response to two injections of 1 Lf

Neither have we been able to confirm the very pronounced decrease in antigenicity by incorporation of glycine reported by the above-mentioned authors The dose response curves both for $Al(OH)_3$ adsorbed and plain toxoids did not disclose significant dissimilar[illegible]

[illegible] toxoid Our results thus seem to be more in accordance with those of *Neumuller* (3), who reported an excellent antigenic effect of glycine toxoid

However, when carrying out an assay by the two-dose method used by *Linggood et al* (2), some decrease in antigenic effect was disclosed, the mean of the antitoxic response being significantly lower for the glycine toxoid than for the lysine and control toxoids The change of the molecule can scarcely be very extensive, since it does not manifest itself significantly in the dose-response curve of single doses

It remains to be shown whether the antigenicity is affected by the conditions used for the amino acid incorporation If so, that may be

the reason for the discrepancy between our results and those of *Linggood et al* (2) as regards the antigenicity

SUMMARY

It is verified that irreversible detoxification of purified diphtheria toxin can only be obtained in the presence of an amino acid Under the experimental conditions used, lysine or alanine and lysine combined were able to ensure a completely stable toxoid Alanine alone and glycine did not yield irreversible toxoids but decreased the reversal degree very markedly as compared to toxoids detoxified without amino acids

With lysine, an irreversible outcome is dependent both on the actual and the relative concentrations of HCHO and lysine, and on pH For some ratios, stable toxoids have been obtained over a pH range from 6 4 to 7 6, whilst for others the pH range is much narrower

The stabilizing effect of lysine is almost independent of the duration of previous contact between HCHO and toxin and can be obtained even when the toxin has been completely detoxified previously

Only small and unsystematic decreases in flocculating values have been observed both during detoxification under the varying conditions applied and during the reversal testing

Incorporation of lysine did not change significantly the antigenicity of the toxoid tested, either when applied in $Al(OH)_3$-adsorbed or in plain form Incorporation of glycine caused some reduction in the antigenicity This could not be demonstrated with the $Al(OH)_3$-adsorbed toxoid, and only under certain conditions with the plain toxoid

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THE IMPORTANCE OF THE DURATION OF INCUBATION IN THE INVESTIGATION OF BLOOD CULTURES

By

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Great variations exist in the techniques which are used to determine whether or not bacteria are present in the blood obtained from patients. In addition to differences in the methods of disinfecting the skin, there are also variations in the transport time and in the medium used for transportation. In the bacteriological laboratories variations are found in the nature of culture media and the number used for each test, in the the duration of incubation, and in the criteria which are accepted as necessary for the demonstration or exclusion of growth. These last may consist of inspection of the culture for growth, microscopy of unstained or stained preparation, and inoculation onto solid media.

This state of affairs, in which the procedures used in blood culture vary so greatly that it is difficult to find two bacteriological laboratories which use the same methods (*Kolin* 1952), has presumably arisen because each procedure has been developed on the basis of experience in daily routine and not on the basis of investigations designed to reveal the best method.

The author of the present article has been unable to find any publication in the literature referring to investigations into the importance of the duration of the incubation.

For ordinary routine blood culture, that is when the culture is not especially intended to reveal the presence of particularly slowly growing bacteria such as, for example, *Brucella*, the available text books of bacteriological technique recommend greatly differing incubation periods, but in no case is reference made to any published investigation of the matter.

The usual duration recommended is two weeks (*Schiødt* 1931, *Kolin* 1952, *Lofstrom & Ericsson* 1953, *Isenberg & Berkman* 1962, *Baily & Scott* 1962) or three weeks (*Stokes* 1960, *Bauer* 1962, *Jawetz et al* 1962), but the times suggested vary from four (*Mackie & McCartney* 1960) or five ... to four weeks (*Butler* 1937).

Fo... ...ne of one week has been used for ... ...uminstitut, as experience has

shown that the results obtained after one week s incubation were equal to those obtained after two weeks (*Tarnowski* 1944)

The purpose of the present work has been to investigate more systematically whether an incubation time of one week is sufficiently long

MATERIAL AND METHODS

The blood samples in venules are sent from all parts of Denmark to the bacteriological diagnostic department of Statens Seruminstitut and may be up to 24 hours in transit On arrival at the laboratory the contents are divided aseptically among *4 tubes of serum broth 4 tubes of semi solid broth agar and 4 thioglycollate tubes* (i e tubes containing semi solid broth to which thioglycolic acid has been added) and all tubes are incubated at 35° C

The tubes are inspected daily in transmitted light and if suspect colonies or other manifestations of growth are observed some of the material is removed and examined for bacteria by microscopy and by inoculation onto solid media If after one week inspection has not revealed any evidence of growth the culture is reported to have shown no growth

For the present *investigation 240 complete sets of 12 tubes* were chosen at random from among those which had been considered to belong t the group no growth after routine culture by the method described above

One third of the sets were examined further on the same day one third were incubated for a total of two weeks and the last group was incubated for a total of three weeks before control examination Thus each group of 80 sets of tubes was examined on only one occasion This method was chosen to obviate the risk of secondary contamination which is always involved when a culture tube is opened The method used for control examination was the same in all cases and consisted in the removal of material fr m the bottom and top of every tube and also from any suspect areas This material was inoculated on blood agar plates and was also examined unstained in a wet mount by phase microscopy

If microscopy revealed suspect or definite bacteria Gram staining was carr ed out

The blood agar plates were incubated aerobically at 35° C for 2 3 days and were examined after this time and then again after they had been allowed to stand at room temperature for 2 3 days

In occasional cases in which possible bacter a were seen on microscopy but no growth was obtained on aerobic culture anaerobic cultures were ma le or it was attempted to subculture on semi solid agar and use ther staining methods

The identification of the bacteria discovered was carried out by the methods usually employed in the laboratory

RESULTS

Growth was found in one set of the 80 one week cultures in *two* sets of the two week cultures and in *three sets of the 80 three-week* cultures

Two different kinds of bacteria were found in one of the sets (Table 1) whilst only one kind of bacteria was found in each of the remaining five sets

The bacteria demonstrated in sets I and III (Table 1) *Alcaligenes faecalis* and unidentified Gram negative rods and Gram positive coryneform rods respectively may be assumed to have been contaminants The bacteria found in set II (Table 1)—coagulase negative *Staphylococcus albus*—may have indicated a true bacteraemia as the patient was severely debilitated by Hodgkin s disease The last three positive findings (sets IV V and VI in Table 1) of coagulase-positive *Staphylococcus aureus* and non haemolytic streptococci must all be accepted as

TABLE 1

Incubation period	Specimen No	*Organism isolated*	Positive tubes out of 12	Other bloodcultures from the same pat	Clinical diagnosis	Chemo therapy given
1 week	*I*	1) Alc faecalis 2) *unidentified* Gram negative rods	1 2	0	Fever of unknown origin	0
2 weeks	*II*	Staph alb coag neg	3	No 2 negative	Hodgkin s disease	0
	III	Gram pos corynebact	10	No 1 negative No 2 Staph alb (2/12)* No 4 Staph alb (7/11)	Bronchitis Fever	0
3 weeks	IV	Staph aur coagulase positive	1	No 1 Proteus mirab + *Staph aur* No 2 Proteus mirab No 4 *negative* No 5 Proteus mirab + Staph aur	Perisigmoiditis with perforation to peritoneum	+
	V	Staph *aur coagulase* positive	4	No 2 negative	Fever Tender mass in left gluteal *region* (abscess?)	+
	VI	nonhaemolytic *streptococci*	3	No 1 negative No 2 nonhaemolytic *streptococci* (2/12)	Aortic insuff endocarditis	+

* Number of tubes with growth out of tubes inoculated

shown that the results obtained after one week's incubation were equal to those obtained after two weeks (*Tarnowski* 1944)

The purpose of the present work has been to investigate more systematically whether an incubation time of one week is sufficiently long

MATERIAL AND METHODS

The blood samples in venules are sent from all parts of Denmark to the bacteriological diagnostic department of Statens Seruminstitut and may be up to 24 hours in transit On arrival at the laboratory the contents are divided aseptically among 4 tubes of serum broth, 4 tubes of semi-solid broth agar, and 4 thioglycollate tubes (i e tubes containing semi-solid broth to which thioglycolic acid has been added) and all tubes are incubated at 35° C

The tubes are inspected daily in transmitted light, and if suspect colonies or other manifestations of growth are observed some of the material is removed and examined for bacteria by microscopy and by inoculation onto solid media If, after one week inspection has not revealed any evidence of growth the culture is reported to have shown 'no growth'

For the present investigation 240 complete sets of 12 tubes were chosen at random from among those which had been considered to belong to the group no growth after routine culture by the method described above

One third of the sets were examined further on the same day, one third were incubated for a total of two weeks, and the last group was incubated for a total of three weeks before control examination Thus each group of 80 sets of tubes was examined on only one occasion This method was chosen to obviate the risk of secondary contamination which is always involved when a culture tube is opened The method used for control examination was the same in all cases and consisted in the removal of material from the bottom and top of every tube and also from any suspect areas This material was inoculated on blood agar plates and was also examined unstained in a wet mount by phase microscopy

If microscopy revealed suspect or definite bacteria Gram staining was carried out

The blood agar plates were incubated aerobically at 35° C for 2–3 days and were examined after this time and then again after they had been allowed to stand at room temperature for 2 3 days

In occasional cases in which possible bacteria were seen on microscopy but no growth was obtained on aerobic culture anaerobic cultures were made or it was attempted to subculture on semi solid agar and use other staining methods

The identification of the bacteria discovered was carried out by the methods usually employed in the laboratory

RESULTS

Growth was found in one set of the 80 'one week" cultures, in two sets of the "two-week" cultures, and in three sets of the 80 "three week' cultures

Two different kinds of bacteria were found in one of the sets (Table 1), whilst only one kind of bacteria was found in each of the remaining five sets

The bacteria demonstrated in sets I and III (Table 1), *Alcaligenes faecalis* and unidentified Gram negative rods, and Gram-positive coryneform rods, respectively, may be assumed to have been contaminants The bacteria found in set II (Table 1)—coagulase negative *Staphylococcus albus*—may have indicated a true bacteraemia, as the patient was severely debilitated by Hodgkin s disease The last three positive findings (sets IV, V, and VI in Table 1), of coagulase-positive *Staphylococcus aureus* and non haemolytic streptococci must all be accepted as

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* Number of tubes with growth out of tubes inoculated

indicating bacteraemia in the patients, in sets IV and VI because other blood samples from the same patient also contained the same bacteria and in all three cases because the bacteriological findings were in keeping with the patient's clinical disease

DISCUSSION

The one positive set found at control examination of the 'one week' incubation cultures may be ascribed either to an error of observation during routine examination, or to the fact that the routine examination comprises only inspection of the tubes, whilst the control examination was more comprehensive and included microscopy and culture from all tubes In favour of the first possibility is the fact that after the control investigation had revealed the positive finding among the "one-week incubation sets, the member of the staff who had carried out the rou tine examination was able to point out on renewed inspection the correct positive tube in the relevant set On the assumption that an error of observation may involve that about one positive sample is missing among 80 sets, a discovery of two and three sets with growth, but not previously found among the sets incubated for two and three weeks, respectively, cannot be taken as evidence in support of the theory that an extension by two or three weeks of the incubation period would be sufficient *per se* to give a statistically certain increase in the incidence of positive results On the other hand, it cannot be excluded either that the extension of the incubation time, as such, occasionally might have been the cause of the discovery of the growth Apart from the non haemolytic streptococci, and perhaps also the coryneform rods, all the bacteria found in the "two-week" and "three-week" incubation sets normally grow so rapidly that it seems improbable that no growth was visible at the routine examination after one weeks incubation This again would indicate that the failure to detect growth at the routine examination was due to errors of observation at least in three or four of the five cases

In this department incubation of the samples for one week will continuously be considered sufficient, but a more frequent inspection of the sets is recommended, preferably by different observers Microscopy and subculture from all tubes is impossible in practice but should perhaps be used in special cases, which must be selected on the basis of a clinical-bacteriological co operation

The late development of visible growth could not in all instances be due to the fact that the blood contained chemotherapeutic drugs, as the patients from whom the two positive results in the 'two-week" incubation group originated were not receiving such therapy However, this, might have been the case with some of the positive findings in the "three-week" incubation sets, as these patients received chemotherapeutic drugs

SUMMARY

The routine method of blood culture involving the exclusion of growth solely on the basis of inspection of the cultures after incubation for one week has been controlled by examination of unstained wet-mount by phase microscopy, Gram staining, and inoculation onto blood agar plates

Of 80 specimens which had been considered negative on routine examination one was found to be positive on further examination the same day

Prolongation of the incubation period to 2 and 3 weeks did not give significant increases in the number of positive results

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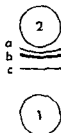


Fig 1

The main agar precipitation lines produced by strain 263 (1) against homologous immune serum (2)

a Polysaccharide A line
b Polysaccharide 263 line
c Antigen D line

strain 263 against homologous immune serum are shown in Fig 1 The *immune sera* were produced by intravenous injections of formalin killed microbes into rabbits (21)

Fractionation Methods

Crude material was prepared as described earlier (15) Live 263 microbes were extracted at 37° C with 0.2 M acetate buffer of pH 5 8 Dilute hydrochloric acid was added to the extract and the precipitate formed at pH 4 2 was removed after which the antigenic material was precipitated with ethanol

Diethylaminoethyl (DEAE) *cellulose* (Eastman) 100 230 mesh particle size was prepared and run on columns as described by *Peterson & Sober* (22) and *Haukenes* (8) respectively Gradient elution was carried out with NaCl and the system was ar [illegible]

[illegible] and the duration 180 min Details of the procedures for application and running of the material have been given earlier by *Haukenes* (12) After electrophoresis the antigenic material was revealed on the paper by eluting strips cut parallel to the application line and by examination of the water eluates by agar precipitation

Paper Chromatography

Circular paper chromatography was carried out on acid and alkaline hydrolysates with Whatman No 1 filter paper as the stationary phase using the method described by *Giri & Rao* (6) and modified by *Grov & Alvsaker* (7) The hydrolysates were prepared as described in (15)

A sample of the antigen D preparation was also hydrolysed for 1 hr at 100° C in an alcoholic solution of N KOH

Spray reagents [illegible] acid (b) Na peri[illegible] *Partridge* (d) an[illegible] and (f) copper ace[illegible] solvent systems a[illegible] [illegible] given previously (15)

Acta path et microbiol scandinav 65 134-142, 1965

The University of Bergen School of Medicine, The Gade Institute
Department of Microbiology, Bergen Norway

STUDIES ON THE ANTIGENIC STRUCTURE OF THE 80/81 COMPLEX OF *STAPHYLOCOCCUS AUREUS*

5 *Preparation of an Antigenic Polysaccharide-Peptide Compound*

By

TOR HOFSTAD

Received 2 iii 65

The precipitinogens found in the *Staphylococcus aureus* strain 263 (phage type 80 81/82/KS6) have been reported in a previous paper (15) Three principal lines were seen when extracts of live or crushed 263 microbes were examined by double diffusion in agar against the homologous immune serum Two of these lines were produced by two ribitol teichoic acid-peptide compounds polysaccharide A, which had earlier been purified by *Haukenes* (9) from another *Staph aureus* strain (Wood 46), and polysaccharide 263, which was purified by the author (15) The chemical basis of the serological reactivity of the compounds was confined to the teichoic acid moieties, being β-N-acetyl-glucosaminylribitol in polysaccharide A and α-N-acetyl-glucosaminylribitol in polysaccharide 263 (12, 16)

The present paper deals with the preparation and some chemical data of the antigen producing the third agar precipitation line, provisionally named antigen D The object has been to prepare the antigen sufficiently purified to form a chemical basis for further studies on its serological reactivity, not to make a detailed chemical study on a 100 per cent purified product

MATERIALS

The bacteria were grown for 18 hrs on
The harvested organisms were either

Ribitol teichoic acid from cell walls of *Staph aureus* strain 3528 (20) (furnished by Professor *J L Strominger* St Louis Mo) *Polysaccharide A* and *polysaccharide 263* were used as reference substances Polysaccharide A was prepared from strain Wood 46 as described by *Haukenes* (9) and polysaccharide 263 from strain 263 KS6 6 as described in a previous paper (15)

α and β glucosidases were obtained from Koch Light Laboratories Colnbrook

METHODS

Agar Precipitation

Double diffusion in agar was performed as described earlier (15) but the incubation was carried out at room temperature The three principal lines produced by

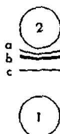


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relative to the application line and by examination of the water eluates by agar precipitation

Paper Chromatography

Circular paper chromatography was carried out on acid and alkaline hydrolysates with Whatman No 1 filter paper as the stationary phase using the method

Spray reagents (a) Ninhydrin 0 4 per cent in acetone containing 2 per cent acetic acid (b) Na periodate benzidine, (c) the Elson Morgan reagent as modified by *Partridge* ... time
and (f) co... the
solvent sy...

Analytical Methods

Phosphorus was determined by the method of *Fiske & Subbarow* (4), but perhydrol was added during digestion instead of nitric acid (25) The *molar ratios of the amino acids* were estimated chromatographically as described by *Gros & Hvsalker* (7)

The qualitative *Molisch* and *biuret* tests were performed as described by *Haukenes* (10)

Optical density was measured in a Unicam SP 500 Spectrophotometer with 1 0 cm cells

EXPERIMENTAL PROCEDURES AND RESULTS

A *Purification of Antigen D*

Crude material was prepared as outlined above (cf methods) The specific antigen D line was obtained in dilutions of the freeze-dried crude material up to 1 5 × 10^2

In the experiment to be described, 2 g of crude material were dissolved in 0 02 M phosphate buffer of pH 7 4, and applied to a column of DEAE cellulose The resin had previously been equilibrated with the same buffer Gradient elution was carried out with NaCl and 10 ml fractions were collected and examined by agar precipitation against 263 serum Antigen D was eluted between NaCl molarities of 0 28 and 0 45 together with some polysaccharide 263 and some polysaccharide A However, the bulk of the polysaccharide 263 and polysaccharide A antigens was eluted below salt concentrations of 0 28 M NaCl (cf Fig 2 in (15)) The optical density of the fractions was recorded at 280 mμ Peaks of ultraviolet light absorbing material were observed in the first fractions and at salt concentrations above 0 5 M but some absorption was also exhibited by the fractions containing antigen D

All fractions containing antigen D were combined, dialysed against tap water overnight, and the volume reduced by distillation under reduced pressure The dialysed and concentrated material was then subjected to re-chromatography on DEAE cellulose until no more polysaccharide 263 and polysaccharide A were eluted at salt concentrations below 0 28 M NaCl The optical density and serological activity of the fractions obtained after the third (and last) re chromatography are shown in Fig 2

The antigen D containing fractions from the last DEAE cellulose column were combined and dialysed against tap water for 48 hrs The yield of freeze-dried material, *i e* crude antigen D was 140 mg The highest dilution of the freeze dried preparation producing the antigen D line was 1 6 × 10^3 The polysaccharide 263 and polysaccharide A lines were obtained in dilutions of 1 8 × 10^4 and 1 4 × 10^3 respectively Calculated according to the agar precipitation titres of the purified polysaccharides 263 (1 10^6) and A (1 10^6), the contaminating polysaccharide antigens contributed approximately to 10 per cent of crude antigen D

Gel filtration through Sephadex and paper electrophoresis were tried

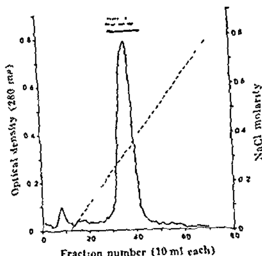


Fig 2
Third re-chromatography of antigen D on DEAE cellulose
Polysaccharide A
— — — Polysaccharide 263
——— Antigen D
– – – Effluent molarity

for further purification of antigen D When eluted with water or 0 02 M phosphate buffer of pH 7 4, antigen D went straight through columns of Sephadex G-50, G-75, G 100 and G-200 together with the bulk of the contaminating polysaccharides Some polysaccharide 263 and polysaccharide A were also found on further elution, particularly from Sephadex G 100 and G-200

By paper electrophoresis (Whatman No 1 paper) in veronal and acetate buffers of pH 8 6 and 5 8 respectively, all three antigens migrated towards the anode and could be eluted from the same areas of the paper With N acetic acid of pH 2 3 a definite separation of antigen D from polysaccharide 263 and polysaccharide A was obtained While polysaccharide 263 and polysaccharide A migrated 14 to 22 cm towards the anode in 3 hrs at the voltage used, antigen D remained on the line of application A complete separation was only obtained, however, when 2 mg or less of crude antigen D was applied to the paper (15 × 30 cm) When greater amounts of the crude antigen preparation were applied, minute amounts of polysaccharide 263 were found on the line of application together with antigen D The recovery after electrophoresis was not fully quantitative, as judged by titre Because of the small amounts obtained on each electrophoresis, no attempts were made to obtain the purified antigen as a freeze-dried compound The further chemical examinations were all made on aqueous eluates

B *Some Chemical Data of Antigen D*

Crude antigen D was obtained as a yellowish, granular powder after freeze-drying The crude antigen was slightly soluble in buffers of neutral or alkaline pH but dissolved readily at an acid pH, particularly in N acetic acid

The Molish test was positive when carried out on a 0 1 per cent aqueous solution of crude antigen D The same solution showed a negative biuret test

The crude antigen was rich in phosphorus The figure for the present preparation was 8 1 per cent, but somewhat lower contents have been found in two other batches of crude antigen D

The U V absorption spectra of crude and purified antigen D were similar to that of polysaccharide 263 (cf Fig 3 in (15)), *i e* showing a slow and continous fall from 240 to 300 mμ, without any peaks

Paper Chromatography

Two N and 3 N hydrochloric acid hydrolysates of crude and purified antigen D were examined for sugar alcohols and neutral sugars in system (C) The hydrolysates of *purified* antigen D produced only one spot on sodium periodate benzidine treated chromatograms, and this was identified as glycerol The glycerol spot was very strong and could be obtained with small amounts of the hydrolysates which produced no amino acid or glucose spots (cf below) The hydrolysates of crude antigen D produced weak ribitol and anhydroribitol spots in addition When the hydrolysates of crude and purified antigen D were examined for neutral sugars, an aldohexose, identified as glucose in system (D), was demonstrated The glucose spot was rather weak

Samples of crude antigen D were also subjected to paper chromatography after treatment with α- and β-glucosidases The enzymatic treatment was carried out at 37° C for 18 hrs in buffered solutions of pH 6 8 (α-glucosidase) and pH 5 0 (β glucosidase) The treatment did not release any glucose

The 3 N hydrochloric acid hydrolysates were examined for amino sugars in the systems (A) and (C) While glucosamine was identified with both the ninhydrin and the modified Elson Morgan reagent in hydrolysates of crude antigen D, no glucosamine (or muramic acid) could be demonstrated in several hydrolysates of the purified antigen

Examination for amino acids was carried out by running 6 N hydrochloric acid hydrolysates of crude and purified antigen D in systems (A) and (B) Glutamic acid serine, glycine, alanine, aspartic acid, lysine, and traces of valine and leucine were identified with ninhydrin The molar ratios relative to lysine of the six principal amino acids found in the purified antigen D glutamic acid serine glycine alanine, aspartic acid lysine, were 5 4 3 2 2 1

An alkali hydrolysate of purified antigen D (N NH_4OH for 5 min at

100 C) was examined for amino acids in system (C) Weak alanine and serine spots were found on ninhydrin treated chromatograms An alkali hydrolysate of ribitol teichoic acid from cell walls of strain 3528 produced a strong alanine spot

The sample of crude antigen D hydrolysed with the alcoholic solution of N KOH were examined for fatty acids in system (F), using Whatman paper No 1, which had been pre-treated with a solution of 10 per cent paraffin in ether No spots were obtained by treatment of the dried chromatogram with copper acetate-potassium ferrocyanide

Nucleic acid derivatives were not found when the 0 1 N hydrochloric acid hydrolysate was examined in system (E)

The products of acid hydrolysis of crude and purified antigen D have been compiled in Table I The products obtained on acid hydrolysis of polysaccharide 263 have been listed for comparison

TABLE 1

Products of Acid Hydrolysis of Crude and Purified Antigen D and of Polysaccharide 263

Products	Antigen D		Polysaccharide 263*
	Crude	Purified	
Glycerol	+	+	—
Ribitol	+	—	+
Anhydroribitol	+	—	+
Glucose	+	+	—
Glucosamine	+		+
Glycine	+	+	+
Alanine	+	+	+
Lysine	+	+	+
Glutamic acid	+	+	+
Serine	+	+	—
Aspartic acid	+	+	—
Valine	tr	tr	—
Leucine	tr	tr	—

* Data from (15) tr = trace amounts

DISCUSSION

The product obtained after fractionation on DEAE cellulose columns, i e crude antigen D, was contaminated with polysaccharide 263 and polysaccharide A The contaminating antigens [illegible]

[illegible] amounts of the crude antigen were applied to the paper

That the crude and purified antigen D preparations were contaminated with serologically inactive material cannot be excluded The high optical density of the antigen containing fractions from the last re-

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An alkali hydrolysate of purified antigen D (N NH_4OH for 5 min at

minary examinations of isolated cell walls from strain 263 indicate that antigen D is a wall component (18)

The amino acids glutamic acid, serine, glycine, alanine, aspartic acid, lysine, and trace amounts of valine and leucine were found in all acid hydrolysates of the different antigen D preparations examined If these amino acids represent one particular molecular unit, which however has not been proved, this peptide is therefore different from the peptide moiety of the simultaneously extracted polysaccharide 263

SUMMARY

A precipitinogen has been isolated from extracts of the *Staphylococcus aureus* strain 263 The antigen was purified by gradient elution on diethylaminoethyl (DEAE) cellulose columns and electrophoresis at pH 2 3

Chemical examinations indicated that the prepared product was made up of alanine-free glycerol teichoic acid containing some glucose residues, and peptide

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chromatography on DEAE cellulose (cf Fig 2) may support this On the other hand, the absorption may well be due to the elution of large amounts of the antigens The largest amounts of antigen D were particularly found in the fractions showing the highest extinction Polysaccharide A and polysaccharide 263 both exhibit some absorption at 280 mμ (10, 15), and it is reasonable to believe that this is also true for antigen D

In spite of the uncertainty about the purity of the antigen D preparations, the results obtained by the chemical examinations carried out gave a clear indication of the qualitative composition of the antigen As the chemical composition of polysaccharide A and polysaccharide 263 is known, valuable information was also obtained by examination of the crude antigen D preparation

The outstanding findings were the high phosphorus content of crude antigen D and the great amounts of glycerol in the acid hydrolysates of of the crude and purified antigen As nucleic acid derivatives and fatty acids were not demonstrated in the appropriate hydrolysates, the phosphorus cannot be derived from nucleic acids or from phospholipids in the cytoplasmic membrane The phosphorus is, therefore, in all probability bound to glycerol as glycerophosphate It is likely that glucose is attached to the glycerophosphate (1), although no release of free sugar was obtained by treatment with α- or β glucosidases

The findings of amino acids in addition to the other components, indicate that antigen D is made up of two structural units a glycerophosphate polymer containing some glucose residues, *i e* a teichoic acid, and a peptide The teichoic acid moiety is, most likely, lacking ester linked alanine

Antigen D thus seems to be a structure similar to the simultaneously extracted polysaccharide 263 and polysaccharide A antigens This assumption is in good agreement with the similar behaviour of the three antigens on columns of DEAE cellulose and Sephadex Analogous to polysaccharide A and polysaccharide 263, recent investigations suggest that the presumed teichoic acid moiety of antigen D constitutes the chemical basis of the serological reactivity (17)

Serologically active polyglycerophosphate has been prepared from *Staph aureus* by *McCarty* (19) The antigen contained no sugar and could also be prepared from group A streptococci and from other Gram positive bacteria

RajBhandary & Baddiley (23) have isolated "intracellular' glycerol teichoic acid containing small amounts of glucose residues from ribosomal fractions of *Staph aureus H* It is not known whether this teichoic acid is serologically active Investigations by *Hay et al* (13) suggest that the "intracellular' teichoic acid is located outside and close to the cytoplasmic membrane Small amounts of glycerol teichoic acids have also been found in cell wall preparations of *Staph aureus* (1) These investigations are of interest to the present work since preli-

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STUDIES ON THE ANTIGENIC STRUCTURE OF THE 80/81 COMPLEX OF *STAPHYLOCOCCUS AUREUS*

6 *Antigenic Properties of a Polysaccharide-Peptide Compound*

By

TOR HOFSTAD

Received 2 iv 65

In a previous paper (14) the purification of antigen D, a precipitinogen from the *Staphylococcus aureus* strain 263, was described Chemical analyses of the preparation indicated that it was composed of glycerophosphate containing some glucose residues, and a peptide

The present paper deals with the serological behaviour of antigen D, and with studies on the chemical basis of the serological reactivity The distribution of the antigen among staphylococcal strains has also been examined

MATERIALS

Strains [illegible]
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tests and [illegible] colonies on blood agar plates

Strains belonging to the following *Micrococcus* groups were used *M luteus M roseus M conglomeratus M varians, M lylicus* and *M violagabriellae* These strains were obtained from the Czechoslovak Collection of Microorganisms (CCM), Brno, by the courtesy of Dr *M Kocur*

[illegible] *osnegard & Oeding* (15) and [illegible] *aride SPA* (4) was provided

[illegible] *immune serum were obtained*

METHODS

Agar precipitation and *ring test precipitation* were carried out as described earlier (12 13) Incubation was performed at room temperature

14 *Hofstad T* Studies on the antigenic structure of the 80/81 complex of *Staphy lococcus aureus* 1 Agglutinogens Acta path et microbiol scandinav *61* 558 570 1964
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overnight at 4° C, and examined by agar precipitation against antigen D Inhibition was not demonstrated

Complement Fixation

Complement binding antibodies against antigen D were found in all homologous sera and in immune sera prepared against our type strains Positive reactions were obtained with dilutions of purified antigen D up to 1 100 of the highest dilution giving the specific agar precipitation line Treatment of the immune serum with mercaptoethanol did not effect the complement fixation

The complement fixation titres using preparations of antigen D, polysaccharide A and polysaccharide 263 as antigens, were then compared (Table 1) The results indicate that complement fixation, like agar precipitation, recognizes three non identical antigens

TABLE 1

Complement Binding Antibodies in Some Staph aureus *Immune Sera*

Antigen	Reciprocal titre values in sera					
	263	365	3617	F 21	CII	CIII
Antigen D*	2560	80	80	80	80	20
Polysaccharide 263 1 µg/ml	640	–	80	–	10	80
Polysaccharide A 0 5 µg ml	40	–	20	20	40	20

Highest dilution giving the precipitation line diluted 1 50

Agglutination Inhibition

Four 263 immune sera, diluted 1 10, were each mixed with equal volumes of a 0 1 per cent solution of crude antigen D and incubated for 2 hrs at 37° C and overnight at 4° C The sera were then examined for agglutinating antibodies against live 263 microbes Inhibition of agglutination was not demonstrated Neither did antigen D show any inhibiting power over the agglutination of 263 microbes in the factor 263–1, 263–2, *m* or a_3 sera (10)

Indirect Haemagglutination

Purified antigen D did not sensitize normal or tanned sheep erythrocytes to agglutination in 263 immune sera Varying amounts of the purified antigen were used for sensitization of 0 1 ml packed cells, and four 263 sera were examined for agglutinating antibodies to the treated sheep cells

The Distribution of Antigen D among Staphylococcal Strains

The antigen D line was produced by the variant strains obtained by *in vitro* lysogenization of strain 263 (11) The specific line was also

The *methods* employed for *complement fixation* and *agglutination* were the same as in (13)

Indirect haemagglutination was performed as described by *Oeding et al* (22) Normal and tanned (1 40 000 of *tannic acid* Merck) sheep erythrocytes were sensitized as outlined by *Morse* (18)

Rabbit immune sera were produced as described by *Oeding* (20) Treatment of immune sera with 2 mercaptoethanol (Fluka) was carried out by adding equal volumes of a 0 2 M *solution of 2* mercaptoethanol in phosphate buffered saline pH 7 4 to samples of undiluted serum (6)

Preparation of factor sera and *antibody absorption* were performed as described in (10)

Treatment with enzymes Enzymatic digestion of antigen D was performed with crystalline trypsin (*Trypure Novo*) crystalline *α* chymotrypsin (Sigma) protease (Nutritional Biochemicals Corporation) pepsin 3 times crystallized (Sigma) and papain (Merck) *The treatment* with trypsin *α* chymotrypsin and protease was carried out at pH *7 8 in* 0 1 M *tris buffer* containing 0 05 M Ca^{++} The treatment with pepsin and papain was carried out in 0 1 M citrate buffer of pH 2 0 and at pH [illegible] in 0 1 M *phosphate buffer* containing 0 01 M cysteine hydrochloride and [illegible] M titriplex II (Merck) respectively The digestions were performed at 37° C for 18 hrs with *an enzyme* substrate ratio of 1 10 (w/w)

Digestion was also performed with a toluol water suspension of crystalline car*boxypeptidase A* (Sigma) The treatment was carried out at 37° C for 4 hrs at pH 8 5 *in 0 1 per cent* sodium bicarbonate buffer The ratio enzyme substrate was 1 50 (w/w)

Treatment with *α* and *β* glucosidases was carried out as described earlier (14)

Oxidation with periodic acid was performed by adding a 0 1 per cent solution of *crude antigen D* in 0 1 M phosphate buffer pH 7 0 to an equal volume of 0 09 M *periodic acid* (1) The mixture was incubated in the dark at room temperature overnight

EXPERIMENTAL PROCEDURES AND RESULTS

Precipitation

The specific antigen D precipitation line against immune serum 263 was produced by crude antigen D to a dilution of 1 6 × 10^{3} Since the purified antigen D was not prepared in a dried state (cf 14) the titre values cannot be given on the basis of the dry weight Solutions of purified antigen D reacted in the ring test and on agar precipitation to identical titres

Precipitating antibodies against antigen D were found in all immune sera against strain 263 With most potent sera the antigen D line could be demonstrated in dilutions up to 1 8 in others only when undiluted The precipitation was not influenced by treatment with mercaptoethanol

When immune sera against our type strains were tested with purified antigen D the specific band of precipitation was obtained with all of them

The specific antigen D line was not produced by polysaccharide 3019 (containing polysaccharides A and C), polysaccharide 1254 (polysaccharide B) protein A or SPA These antigen preparations were also examined for inhibition of the precipitation of antigen D in 263 serum 0 1 per cent saline solutions of each of the four antigens were mixed with an equal volume of 263 serum incubated for 2 hrs at 37° C and

In a series of experiments antigen D was compared by agar precipitation with samples of different glycerol teichoic acids Made up in saline to 0 1 per cent solutions, none of the preparations from the *Staph epidermidis* strains T1, T2, I2 and I3 were serologically active against 263 immune serum The glycerol teichoic acids were also examined for inhibition of the precipitation of antigen D in 263 immune serum Samples of the saline solutions were added to equal volumes of undiluted serum The serum-glycerol teichoic acid mixtures were incubated for 2 hrs at 37° C and overnight at 4° C, and then examined by agar precipitation against antigen D No inhibition could be demonstrated When examined against homologous immune sera the preparations from T2, I2 and I3 produced only a very weak agar precipitation line, while the preparation from T1 was inactive (21) Precipitating and complement-binding antibodies to antigen D were found in immune sera prepared against strains T1 and I2, but not in immune sera against the strains T2 and I3

The glycerol teichoic acid preparation from *Staph aureus* H produced no antigen D line and did not inhibit the precipitation of antigen D in 263 immune serum No cross-reactivity was demonstrated between antigen D and streptococcal Group D antigen, which is a glycerol teichoic acid containing glucose residues (24) Streptococcal Group D antigen produced two lines against its homologous serum

Finally, glycerol and D glucose were examined for haptenic inhibition of precipitation Undiluted 263 serum was mixed with equal volumes of D glucose or glycerol in varying concentrations, incubated for 2 hrs at 37° C and overnight at 4° C, and examined by agar precipitation against antigen D Inhibition was not demonstrated

DISCUSSION

Serological examination of the antigen D preparations revealed that antigen D, besides producing a specific precipitation line in agar, is also active in the complement fixation test However, the serological activity seems to be low compared to that of polysaccharide A and polysaccharide 263 (7, 12)

The ability to bind complement most probably represents another type of reaction in the specific antigen D-anti antigen D system The complement binding principle of antigen D was not identical with those of polysaccharide A and polysaccharide 263, and complement-binding antibodies against the antigen D preparation were found only in sera giving the specific antigen D line The resistance of the precipitating and complement binding antibodies to treatment with mercaptoethanol indicates that both are low molecular antibodies

The inability of the antigen D preparation to inhibit the agglutination of 263 microbes in unabsorbed 263 serum indicates that antigen D is not exposed to the surface of the staphylococcal cell as a major agglutino

produced by suspensions or extracts of our 13 *Staph aureus* type strains and by extracts of strains H, Copenhagen and Oxford

One hundred strains of *Staph aureus,* isolated in our laboratory from routine specimens, were then examined against 263 serum for production of the antigen D line Fifty of the strains belonged to the 80/81 complex, while the others represented different lytic patterns within phage groups I, II and III, together with non-typable strains and strains belonging to type 187 and the mixed group The examination was carried out with suspensions of live cells Strains which did not produce the specific line were reexamined, using extracts made from live cells in 0 2 M acetate buffer of pH 5 8 for several days at 37° C By this procedure the antigen D line could be demonstrated with all but three strains, one of which belonged to the 80/81 complex None of the three negative strains was able to absorb antigen D precipitins

Thereafter, 100 *Staph epidermidis* strains were examined in the same way Eighty seven of the strains produced the antigen D line When tested for absorbing capacity, representatives of the 13 negative strains were not able to remove antigen D precipitins Immune serum 263 was completely exhausted for antigen D precipitins when absorbed with positive strains

Suspensions or extracts of the *Micrococcus* and the *Streptococcus* Group A strains did not produce the antigen D line, and were unable to absorb antigen D precipitins

Studies on the Chemical Basis of the Serological Reactivity

The sensitivity of antigen D to heat was examined by exposing 0 1 per cent solutions of crude antigen D in 0 02 M phosphate buffer of pH 7 4, to 100° and 120° C for varying periods of time The precipitating activity against 263 serum remained unimpaired after 1 hr of boiling, while autoclaving for 2 hrs caused a three fold reduction in the precipitating activity of antigen D, and destroyed its complement binding property Free amino acids and glucose were not demonstrated when the autoclaved solution was examined by paper chromatography

The sensitivity of antigen D to proteolytic enzymes was then examined No reduction in the serological activity was achieved by treatment with trypsin α chymotrypsin, protease, pepsin or papain Nor was any deteriorating effect demonstrated on the serological activity of antigen D after treatment with carboxypeptidase-A

The serological activity of antigen D remained unaffected after treatment with α- and β-glucosidases The treatment did not release any glucose (14)

In another experiment crude antigen D was subjected to oxidation with periodic acid Both the precipitating and complement-binding properties of antigen D, and also of the contaminating polysaccharide A and polysaccharide 263 antigens, were completely lost by this procedure

In a series of experiments antigen D was compared by agar precipitation with samples of different glycerol teichoic acids Made up in saline o 0 1 per cent solutions none of the preparations from the *Staph epidermidis* strains T1, T2, 12 and 13 were serologically active against 263 immune serum The glycerol teichoic acids were also examined for inhibition of the precipitation of antigen D in 263 immune serum Samples of the saline solutions were added to equal volumes of undiluted serum The serum-glycerol teichoic acid mixtures were incubated for 2 hrs at 37° C and overnight at 4° C, and then examined by agar precipitation against antigen D No inhibition could be demonstrated When examined against homologous immune sera the preparations from T2, 12 and 13 produced only a very weak agar precipitation line, while the preparation from T1 was inactive (21) Precipitating and complement-binding antibodies to antigen D were found in immune sera prepared against strains T1 and 12, but not in immune sera against the strains T2 and 13

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The inability of the antigen D preparation to inhibit the agglutination of 263 microbes in unabsorbed 263 serum indicates that antigen D is not exposed to the surface of the staphylococcal cell as a major agglutino-

gen Similar findings have been made for polysaccharide A and polysaccharide 263 (8, 13)

Antigen D is a widely distributed precipitinogen within genus *Staphylococcus* The few strains which did not produce the specific precipitation line were also negative on absorption However, the negative absorption experiments do not completely exclude the existence in these strains of minute amounts of antigen D, blocked by more superficial antigens

The sensitivity of antigen D to periodic acid indicates that the serological activity is associated with its polysaccharide moiety, presumed to be a glycerol teichoic acid Moreover, the restistance of antigen D to treatment with proteolytic enzymes supports this view

McCarty (17) has demonstrated serologically active polyglycerophosphate in Group A streptococci and in several other Gram positive species among them *Staph aureus* and *Staph albus* The inability of the streptococcal Group A strains to absorb antigen D precipitins and to produce the specific precipitation line, shows that antigen D is not identical with this antigen and indicates that glycerol phosphate alone is not responsible for the serological activity of antigen D

An antigen composed of glycerol phosphate and glucose has been isolated from cell walls of a strain of *Staph albus* by *Morse* (19) Hapten inhibition studies indicated that the antigenic determinant was α-linked glucose The antigen reacted by precipitation with homologous and heterologous *Staph albus* immune sera, but not with immune sera prepared against *Staph aureus* strains

No cross-reactivity was demonstrated between antigen D and polysaccharide 1254, the specificity of which may be due to a glycerol teichoic acid containing glucose residues (16)

None of the different glycerol teichoic acids examined was able to inhibit the precipitation of antigen D in 263 immune serum However, the preparation from *Staph aureus* H was an old one which had been stored in solution for two years, and the preparations from the *Staph epidermidis* strains were either inactive or only slightly active against homologous immune sera The possibility of identity between antigen D and one of these teichoic acids cannot, therefore be excluded

SUMMARY

A polysaccharide peptide compound prepared from *Staphylococcus aureus* strain 263 has been examined serologically The preparation produced a specific agar precipitation line and in addition showed complement-binding property

The antigen is widely distributed within genus *Staphylococcus*

The chemical basis of the serological reactivity of the antigen was not settled However, the data obtained pointed to the polysaccharide

moiety made up of glycerophosphate and glucose, as the antigenic determinant grouping

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STUDIES ON THE ANTIGENIC STRUCTURE OF THE 80/81 COMPLEX OF *STAPHYLOCOCCUS AUREUS*

7 *Preparation and Properties of Cell Walls from Strain 263*

By

TOR HOFSTAD

Received 2 iv 65

In previous studies on the antigenic structure of the 80 81 complex of *Staphylococcus aureus* (13–18), the antigenic materials were prepared by extraction of whole cells Whole cells were also used for agglutination This paper deals with the antigenic properties of cell walls isolated from the prototype strain 263 Besides obtaining information on the properties of *Staph aureus* cell walls *per se*, the object has been to investigate the location in the bacterial cell of antigens described in earlier papers

MATERIALS AND METHODS

Strain 263 has been described earlier (13) The bacteria were grown on nutrient agar for 18 hrs at 37° C and harvested by scraping The methods for preparation of *polysaccharide 263* and *antigen D* have been described in (15) and (17) *Polysaccharide A* and *extract 4* (Jensen) were prepared according to *Haukenes* (10) and *Jensen* (22) *Protein A* was obtained from *Grov et al* (8)

Preparation of Cell Walls

The procedures employed for preparation of the cell walls were modified from *Yoshida et al* (31)

D sintegrati

experiments

About 10 g (wet weight) of crushed bacteria were suspended in 100 ml of 0 1 M phosphate buffer of pH 7 4 containing 0 02 M citrate and 0 001 M

the gradient in a tube and centrifuged at 1400 × g for 1 hr The cell walls formed a diffuse white layer in the middle of the tube, while the non disintegrated whole cells were precipitated together with a varying but small amount of walls The white cell wall layer, which was contaminated with a few intact cells was carefully removed with a pipette washed in *the phosphate buffer* and subjected to one or two further density gradient centrifugations The cell walls obtained were then washed twice in distilled water and freeze dried

The centrifugations were carried out at 0° C in an RC-2 Servall centrifuge with the exception of that at 800 × g and the density gradient centrifugations which were carried out in an MSE Refrigerator Centrifuge using a horizontal rotor All other procedures were performed under sufficient ice cooling

The supernatant from the first centrifugation at 15 000 × g *i e* the protoplasm fraction and all subsequent washings were examined for precipitinogens and agglutination inhibition

Trypsin digestion A portion of the prepared walls was digested with crystalline trypsin (Trypure Novo), freshly prepared in 0 1 M tris buffer of pH 7 8 The digestion was carried out at 37° C for 18 hrs using 0 1 mg trypsin per mg of freeze dried walls

Paper Chromatography

Circular chromatography was performed as described by *Giri & Rao* (6) and *Grov & Alvsaker* (7) A few chromatograms were also run by the descending method Acid hydrolysates were prepared as described in (15) A hydrolysate was also prepared with an alcoholic solution of N KOH (1 hr at 100° C)

Solvent systems (A) BuOH HAc H_2O (4 1 1) (B) PhOH H_2O (4 1 w/v) (C) PrOH NH_3(d 0 91) H_2O (6 3 1) (D) BuOH EtOH H_2O NH_3(d 0 91) (40 10 49 1) organic phase (3) (E) EtAc Py H_2O (40 11 6) (F) *Iso PrOH* 2 N HCl (65 35) and (G) Chloroform Acetic acid Medicinal paraffin (5 13 1)

Spray reagents (a) Ninhydrin, 0 4 per cent in acetone containing 2 per cent acetic acid (b) Na periodate benzidine (c) the Elson Morgan reagent as modified by *Partridge* (d) aniline hydrogen phthalate in water saturated butanol, (e) isatine and (f) copper acetate potassium ferrocyanide

Appropriate references for the solvent systems and the spray reagents have been given previously (15 17)

Chemical Analyses

Phosphorus was determined by the method of *Fiske & Subbarow* (2) Perhydrol was added during digestion instead of nitric acid (33)

Rondle & Morgan's method (29) was used for determination of *hexosamines*

Quantitative determination of amino acids was carried out by paper chromatography (5 7)

Serological Methods

The methods for *agar precipitation* and *complement fixation* have been described in earlier papers (15 16)

Agglutination was carried out on slides (13) and in tubes (15) with whole cells or isolated walls as antigen When cell walls were used as antigen in the tube test one volume of a saline suspension of walls in a concentration of 1 5 mg (dry weight) per ml was mixed with two volumes of serum dilution

Indirect haemagglutination was performed as described by *Oeding et al* (25) using normal or tanned (1 40 000 of tannic acid Merck) sheep erythrocytes sensitized as described by *Morse* (23)

Rabbit immune sera against whole cells were produced by intravenous injections of formalin killed microbes as described by *Oeding* (24) Immune sera against isolated cell walls were prepared in the same way except that the immunization was carried out for four weeks instead of three and the walls were not pre treated with formalin Two rabbits were immunized with total doses of freeze dried walls of 4 5 and 7 0 mg respectively and four other rabbits were immunized with 50 60 70 and 110 mg respectively of wet packed walls from another batch

The *factor sera* were prepared as in (13)

Antibody absorption was performed at 37° C for 2 hrs followed by storage at 4° C overnight

EXPERIMENTAL PROCEDURES AND RESULTS

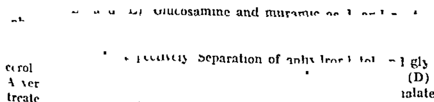
The cell wall preparations were white in colour, although some batches showed a trace of yellow They stained Gram negative with some occasional Gram positive cells Approximately 300 mg of purified walls were obtained from 10 g of crushed microbes Assuming that the wall portion contributes about 20 per cent of *Staph aureus* cells (30), a recovery of aproximately 15 per cent was obtained This rather poor recovery was largely due to inadequate disintegration some cell walls were also lost during the density gradient centrifugations

Four mg of the prepared walls were extracted with 4 ml of 5 per cent trichloroacetic acid for 20 min at 90° C (4) whereafter the extract was examined for ultraviolet light absorption The optical density at 265 mμ was 0 05 The absence of nucleic acids in the preparation was confirmed by examining a 0 1 N hydrochloric acid hydrolysate by paper chromatography in system (F) No spots were detected by examination of the dried chromatogram under the U V lamp

The alkali hydrolysate of the walls were examined for fatty acids in system (G) using Whatman filter paper No 1 which had been pretreated with a solution of 10 per cent paraffin in ether No spots were revealed by treating the dried chromatogram with copper acetate-potassium ferrocyanide

A *Chemical Composition of Strain 263 Cell Walls*

Hydrolysates of the purified walls made with 6 N hydrochloric acid were examined chromatographically for amino acids in systems (A) and (B) Bands corresponding to glycine lysine glutamic acid valine leucine aspartic acid (trace) and serine (trace) were detected The same amino acids except aspartic acid were found in samples of trypsin digested walls Bands corresponding to proline or h



... Glucosamine and muramic ... Separation of ... gly ... cerol ... (D) ... A ver ... treate ... ialate

The results of the quantitative analyses have been compiled in Table 1

B *Antigenic Properties of Strain 263 Cell Walls*

Agar precipitation

Suspensions and extracts of the prepared walls produced the same three main lines against immune serum 263 as whole 263 microbes *i e*

the gradient in a tube and centrifuged at 1400 × g for 1 hr The cell walls formed a diffuse white layer in the middle of the tube while the non disintegrated whole cells were precipitated together with a varying but small amount of walls The white cell wall layer which was contaminated with a few intact cells was carefully removed with a pipette washed in the phosphate buffer and subjected to one or two further density gradient centrifugations The cell walls obtained were then washed twice in distilled water and freeze dried

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The methods for *agar precipitation and complement fixation* have been described in earlier papers (15 16)

Agglutination was carried out on slides (13) and in tubes (15) with whole cells or isolated walls as antigen When cell walls were used as antigen in the tube test one volume of a saline suspension of walls in a concentration of 1 5 mg (dry weight) per ml was mixed with two volumes of serum dilution

Indirect haemagglutination was performed as described by *Oeding et al* (25) using normal or tanned (1 40 000 of tannic acid Merck) sheep erythrocytes sen sitized as described by *Morse* (23)

Rabbit immune sera against whole cells were produced by intravenous injections of formalin killed microbes as described by *Oeding* (24) Immune sera against iso lated cell walls were prepared in the same way except that the immunization was carried out for four weeks instead of three and the walls were not pre treated with formalin Two rabbits were immun zed with total doses of freeze dried walls of 4 5 and 7 0 mg respectively and four other rabbits were immunized with 50 60 70 and 110 mg respectively of wet packed walls from another batch

The *factor sera* were prepared as in (13)

Antibody absorption was performed at 37° C for 2 hrs followed by storage at 4° C overnight

antigen D The double line produced by protein A against Cowan I serum (8) was seen with one of the cell wall immune sera The other cell wall sera gave only one line with purified protein A This line fused completely with the line produced by protein A against pooled human serum A weak polysaccharide 263 line was produced by two sera and the polysaccharide A line by one of the sera Pre immune sera gave no precipitation lines against the prepared walls or against the staphylo coccal antigens mentioned above

The cell wall immune sera contained agglutinating antibodies against the prepared walls and against homologous and heterologous live microbes The highest serum dilution which agglutinated walls or whole 263 microbes varied from 1 400 to 1 1600 Some heterologous *Staph aureus* strains agglutinated to similar titres while others agglu tinated only in dilutions up to 1 40 The sera were also examined for antibodies against the type agglutinogens 263 1, 263–2 *m* and a_2 With the exception of two sera one containing very weak 263 1 agglutinins and the other weak *a* agglutinins antibodies to the type agglutinogens were not detected

TABLE 2

Inhibiting Power of Protein A on the Agglutination of Whole Microbes in Immune Sera Prepared against Cell Walls of Staphylococcus aureus *Strain 263*

Antigen	Reciprocal titres of non absorbed sera and of sera absorbed with protein A					
	K952		K960		K963	
	non ab- sorbed	ab sorbed	non ab- sorbed	ab- sorbed	non ab- sorbed	ab sorbed
Staph aureus strain 263	1280	40	640	40	640	40
Staph aureus strain Cowan I	1280		320	80	1280	20
Staph aureus strain Cowan II	1280		640	40	1280	40
Staph aureus strain 3647	80		40	20	80	
Staph aureus strain 1503	160		40		80	

Portions of cell wall sera diluted 1 10 were mixed with equal volumes of 0 1 per cent saline solutions of protein A or crude antigen D and with 0 01 per cent solutions of polysaccharide A or polysaccharide 263 respectively After incubation for 2 hrs at 37° C and overnight at 4° C the sera were centrifuged in case precipitates had formed and examined by agglutination in tubes against whole staphylococcal cells A marked inhibition of agglutination was obtained with protein A (Table 2) A slight absorption was also obtained with polysaccharide A The polysaccharide A preparation used was later shown to contain minute amounts of protein A No inhibition was recorded with poly saccharide 263 or antigen D Examination of the antigen serum mix tures by double diffusion in agar against non absorbed serum showed that excess antigen had been used in the inhibition test

Complement fixing antibodies against polysaccharide 263 and poly

the polysaccharide 263 line, the polysaccharide A line and the antigen D line These lines were also produced by trypsin digested walls With heavy suspensions, a hardly visible protein A line was produced against pooled human serum

TABLE 1
Main Composition of 100 mg Cell Wall Material from* Staphylococcus aureus *Strain 263*

	Mg	μ Moles	Molar§ ratio
Glycine	20‖	268	3 4
Alanine	13	150	2
Lysine	15	104	1 3
Glutamic acid	11	78	1
Leucine	1	8	0 1
Valine	1	9	0 1
Hexosamine (as glucosamine)	21		
Ribitol phosphate†	17		

* In addition to the components listed traces of serine aspartic acid glucose and glycerol were found by paper chromatography
§ Moles per mole of glutamic acid
† Calculated from the phosphorus content (2 3 mg)
‖ The values listed have not been corrected for water uptake during hydrolysis

Agglutination

The cell walls were agglutinated by 263 serum (prepared against whole microbes) in dilutions up to 1 6400 in the tube test Similar agglutinin titres were obtained with immune sera against heterologous *Staph aureus* strains

On absorption with cell walls, 263 serum was completely exhausted for agglutinating antibodies against whole 263 microbes The absorbing dose was twice the dose of whole 263 bacteria (mg for mg, dry weight) needed for exhaustion of a similar sample of the same serum

The agglutinating activity of the walls varied somewhat from one batch to another Some preparations showed a great tendency to spontaneous agglutination, particularly after freeze drying Trypsinized walls agglutinated only to 1/8 to 1/2 of the titres obtained with undigested walls, varying from one serum to another

The walls did not agglutinate in the factor 263-1 263-2, *m* and *a*, sera, either by slide agglutination or in the tube test On absorption with cell walls, however, the factor sera were completely exhausted for agglutinating antibodies against whole 263 bacteria

Examination of the Cell Wall Immune Sera

The immune sera prepared against the purified walls were examined for precipitating, agglutinating and complement-binding antibodies, and for antibodies agglutinating sensitized sheep erythrocytes

On agar precipitation all sera produced a heavy line against purified

antigen D The double line produced by protein A against Cowan I serum (8) was seen with one of the cell wall immune sera The other cell wall sera gave only one line with purified protein A This line fused completely with the line produced by protein A against pooled human serum A weak polysaccharide 263 line was produced by two sera, and the polysaccharide A line by one of the sera Pre immune sera gave no precipitation lines against the prepared walls or against the staphylococcal antigens mentioned above

The cell wall immune sera contained agglutinating antibodies against the prepared walls and against homologous and heterologous live microbes The highest serum dilution which agglutinated walls or whole 263 microbes varied from 1 400 to 1 1600 Some heterologous *Staph aureus* strains agglutinated to similar titres while others agglutinated only in dilutions up to 1 40 The sera were also examined for antibodies against the type agglutinogens 263-1, 263-2 *m* and *α*3 With the exception of two sera one containing very weak 263 1 agglutinins and the other weak *α* agglutinins antibodies to the type agglutinogens were not detected

TABLE 2

Inhibiting Power of Protein A on the Agglutination of Whole Microbes in Immune Sera Prepared against Cell Walls of Staphylococcus aureus *Strain 263*

Antigen	Reciprocal titres of non absorbed sera and of sera absorbed with protein A					
	K952		K960		K963	
	non absorbed	absorbed	non absorbed	absorbed	non absorbed	absorbed
Staph aureus strain 263	1280	40	640	40	640	40
Staph aureus strain Cowan I	1280		320	80	1280	20
Staph aureus strain Cowan II	1280		640	40	1280	40
Staph aureus strain 3647	80		40	20	80	
Staph aureus strain 1503	160	–	40		80	

Portions of cell wall sera diluted 1 10 were mixed with equal volumes of 0 1 per cent saline solutions of protein A or crude antigen D and with 0 01 per cent solutions of polysaccharide A or polysaccharide 263 respectively After incubation for 2 hrs at 37° C and overnight at 4 C the sera were centrifuged in case precipitates had formed and examined by agglutination in tubes against whole staphylococcal cells A *marked* inhibition of agglutination was obtained with protein A (Table 2) A slight absorption was also obtained with polysaccharide A The polysaccharide A preparation used was later shown to contain minute amounts of protein A No inhibition was recorded with polysaccharide 263 or antigen D Examination of the antigen serum mixtures by double diffusion in agar against non absorbed serum showed that excess antigen had been used in the inhibition test

Complement fixing antibodies against polysaccharide 263 and poly

saccharide A were found in two of the cell wall immune sera All sera contained complement-binding antibodies to antigen D and protein A However, the titres obtained with the different cell wall sera were considerably lower than those obtained with immune sera against whole 263 microbes

The cell wall immune sera were examined for antibodies agglutinating tanned sheep cells sensitized with polysaccharide 263, polysaccharide A or extract A Doses of 0 2 mg of polysaccharides 263 and A, and 0 4 mg of extract A were used for sensitization of 0 1 ml packed cells The sensitized cells were agglutinated by three of the cell wall immune sera The haemagglutinin titres varied according to the antigen used for sensitization of the tanned cells, ranging from 1 40 (polysaccharide A) to 1, 320 (extract A) and 1. 1280 (polysaccharide 263)

The cell wall sera were also examined for antibodies agglutinating normal sheep erythrocytes sensitized with extract A The sensitized cells agglutinated to very low titres (1 10 and 1 40) in two of the six sera examined

C *Examination of the Protoplasm Fraction and Wash Waters*

Heavy polysaccharide 263 and polysaccharide A lines and a weaker antigen D line were found when the protoplasm fraction was examined *by double diffusion in agar against 263 immune serum* The protein A line was not demonstrated A weak polysaccharide 263 line was also found in the different washings

The protoplasm fraction was also examined for inhibition of agglutination of live 263 microbes in 263 serum and factor 263 1, 263-2, *m* and a_5 sera No inhibition was demonstrated

DISCUSSION

When bacterial cell walls are prepared for investigation of their antigenic properties, it is essential that the degradation during the preparation procedure is reduced to a minimum This condition is not fulfilled by the procedures commonly used for preparation of cell walls, which include prolonged shaking with glass beads and digestion with proteolytic and other enzymes under rising temperatures In the present study, crushing of frozen bacteria was preferred to mechanical shaking all further procedures were performed at 0° C, and treatment with enzymes was omitted Satisfactory separation of cell walls and non-disintegrated cells was obtained by density gradient centrifugation The loss of cell wall precipitinogens which nevertheless occurred is due to enzymatic degradation from muralytic enzymes, which could probably only have been prevented by heating the intact cells to 100° C before crushing them

Disintegration in the Hughes' press has been reported to give cell

wall membrane fractions rather than simple wall preparations (20) The negative findings when checked for fatty acids indicate that the wall preparations from strain 263 were not contaminated with cytoplasmic membranes

Chemical analyses of wall hydrolysates revealed the same major cell wall components as found in other *Staph aureus* strains (30), *i e* organic phosphorus, ribitol, glucosamine, muramic acid, and the amino acids glycine, lysine, alanine and glutamic acid The demonstration of small amounts of valine, leucine, aspartic acid and serine has also been reported by other authors (9, 21) Glycerol and glucose have not been reported in *Staph aureus* cell walls prepared by other methods (30) However both components are constituents of staphylococcal "intracellular teichoic acid (27), the cellular location of which seems to be close to though outside the cytoplasmic membrane (12) Small amounts of glycerol teichoic acids have also been demonstrated in trichloroacetic acid extracts of *Staph aureus* cell walls (1)

The data obtained by chemical analyses, including the molar ratios of the four principal amino acids, correspond well to those given for other *Staph aureus* strains (23, 26, 30)

The prepared walls were serologically active on agar precipitation and in agglutination tests The titres obtained by tube agglutination were similar to those obtained by using whole 263 microbes as antigen The walls contained the same agglutinogens as whole 263 microbes, as evidenced by their capability to exhaust the immune sera against whole 263 microbes for agglutinating antibodies However, some degradation seems to have taken place during cell wall preparation, as the walls, contrary to whole 263 microbes, did not agglutinate in the factor 263–1, 263–2 *m* and a_3 sera

The cell wall immune sera agglutinated whole staphylococcal cells and cell walls to lower titres than did immune sera prepared against whole microbes Most of them lacked the type agglutinins and precipitating and complement binding antibodies against polysaccharide 263 and polysaccharide A All cell wall sera contained antibodies against protein A and antigen D The finding of only one agar precipitation line against the protein A preparation in all but one sera, indicates that the double line produced by protein A is due to two different antigenic entities

Earlier studies on the chemical composition and chemical basis of the serological reactivity of polysaccharide A and polysaccharide 263 have shown them to be wall antigens (11, 16) The production of the [illegible] of cell walls, and the regular precipitating and complement- [illegible] that this antigen also is located in the cell wall The demonstration in the wall preparations of glycerol and glucose, which make up the polysaccharide moiety of antigen D (17), is in keeping with this statement

The marked agglutination inhibition caused by protein A shows that this antigen is the major group agglutinogen (or one of the major group agglutinogens) in *Staph aureus* The same finding has been made by *Yoshida, Mudd & Lenhart* (32) It is reasonable to believe, therefore that protein A, analogous to the M protein of Group A streptococci, constitutes the outer layer of the *Staph aureus* cell wall

The demonstration of antibodies in some cell wall sera agglutinating tanned sheep cells sensitized with polysaccharide 263 and polysaccharide A, indicates that the sensitizing principles of these polysaccharides are wall antigens No conclusions can be drawn about the chemical grouping(s) of extract A responsible for sensitization of normal sheep cells, since antibodies to this antigen were demonstrated in very low titres and only in two cell wall immune sera

SUMMARY

Undigested cell walls have been prepared from *Staphylococcus aureus*, strain 263 Chemical analyses of wall hydrolysates revealed the following components organic phosphate, ribitol, glucosamine, muramic acid glycine, alanine, lysine and glutamic acid, *i e* the wall components commonly found in other *Staph aureus* strains Small amounts of valine and leucine were also demonstrated together with traces of aspartic acid, serine, glycerol and glucose

The prepared walls were serologically active on agar precipitation and in agglutination tests Immune sera prepared against them contained agglutinating, precipitating and complement binding antibodies, and antibodies reacting with sensitized tanned sheep erythrocytes The serological examinations showed that the precipitinogens of strain 263—polysaccharide A, polysaccharide 263, antigen D and protein A—were all wall antigens Protein A exhibited a marked inhibition of the agglutination of whole microbes in the cell wall immune sera

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THE ASSOCIATION OF PEPTIC ULCER WITH CIRRHOSIS OF THE LIVER

An Analysis of an Autopsy Series

By

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It is widely believed that peptic ulcer often is found in patients with cirrhosis of the liver This belief seems to be supported by studies of autopsy series (*Schnitker & Hass* 1934 *Ask Upmark* 1940, *Gordon & Manning* 1941 *Puccini* 1950 *Lipp & Lipsitz* 1952 *Fainer & Halsted* 1955 *Watkinson* 1962) as well as of clinical series (*Ratnoff & Patnek* 1942 *Palmer & Brick* 1953 *Swisher Baker & Bennet* 1955 *Fainer & Halsted* 1955 *Koide Texter & Borden* 1958) The evidence of a positive correlation between the two diseases however is far from conclusive because of the special problems of selection involved in studies of association of diseases in hospital data

In the present study a new effort was made to study the association of Laennec s cirrhosis and peptic ulcer in stomach and/or duodenum in an autopsy series in spite of these selection problems

MATERIAL AND METHODS

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... all the cases of Laennec s cirrhos ...

Statistical

general population from which the hospital sample is drawn This principle for which *Mainland* (1953) has proposed the name 'Berkson's Fallacy' is also applicable to autopsy data (For a recent survey see *Grosse* (1964)) If in an autopsy series two disease groups are compared as to the frequency of occurrence of an uncorrelated third disease, a spurious association may arise between this third disease and the disease group with the lower fatality rate *Juhl* (1955) has discussed this fallacy in this journal He tried to eliminate its effect by dividing his series according to the direct cause of death Both on practical and theoretical grounds it seems doubtful whether this method is of any help Even if such a division can be made—and in cases with co existing diseases it may often be far from easy to decide which disease was the cause of death—this method assumes the different fatality rates to operate independently As pointed out by *Berkson* (1946) in his original article this assumption is an oversimplification In the present investigation a different method was used (*van der Linden* 1961)

According to this method, if an association is studied between two disease groups x and y in an autopsy series the cases of x with and without y are classified as mild and severe This gives two groups with different fatality rates A comparison of mild and severe cases of x regarding the frequency of y is also biased by Berkson's competing selection rates which results in a spurious association of y with mild cases of x On the other hand, if there is a true causal relationship between the two diseases y should be more common when x runs a more severe course, and vice versa. Therefore, if y is found to occur more often among the severe cases of x the association may be regarded as true as it exists in spite of the bias of this comparison

ANALYSIS

The autopsy series during the years 1957–1963 consisted of 8114 cases In this series there were 219 cases of Laennec's cirrhosis, in 47 of which peptic ulcers and/or scars after such ulcers were found The ulcers were situated in the stomach in 35 and in the duodenum in 9 cases, in 3 cases ulcers were found both in the duodenum and in the stomach Furthermore, there were 64 cases with cirrhosis of the liver plus primary liver cancer, peptic ulcer being found in the stomach in 13 cases and in the duodenum in one Finally there were 47 cases of primary cancer of the liver without signs of cirrhosis with co existing peptic ulcer in 4 Among the remaining 7784 cases there were 622 with peptic ulcer

Peptic ulcer proved more common in cases with cirrhosis of the liver than in those without This difference did not seem to be due to differences of age or sex It may be reasoned that as patients with cirrhosis can hardly be assumed to have lower death rates than people without, the positive correlation found cannot be a statistical artefact

The autopsy series analysed is, however, not an ideal autopsy sample into which all dead have the same chance of being drawn, irrespective of the disease from which they have died It is the autopsy series of a hospital and even in a community where hospital treatment is free, the chance of dying in hospital may be different for patients with different diseases, depending upon the character of the disease There are a number of reports of possible negative correlations of hepatic cirrhosis with such diseases as extrahepatic cancer (*Fisher, Hellstrom & Fisher* 1960) and myocardial infarction (*Ruebner, Miyai & Abbey* 1961) Now, if a disease x is negatively correlated with a number of diseases with a high risk of dying in hospital, the chance of being drawn into the autopsy

sample may well be smaller for people with x than for people without. In that case the positive correlation found could be a statistical artefact.

Therefore we decided not to content ourselves with the finding of a positive correlation but continued our analysis.

As a first step in our analysis we compared the cases of cirrhosis with primary liver cancer with cases of primary cancer of the liver without cirrhosis (Table 1).

TABLE 1

Incidence of Peptic Ulcer in Cases of Primary Cancer of the Liver with and without Cirrhosis

	Peptic ulcer		
	+	—	Σ
Cancer of the liver without cirrhosis	4	43	47
Cancer of the liver with cirrhosis	14	50	64
	18	93	111

$\chi^2 = 2.65$ d.f. = 1 $0.20 > P > 0.10$

Table 1 shows that peptic ulcer occurred somewhat more often in cases of cancer of the liver with cirrhosis than in cases without. Though no exact information is available about the fatality rates of the two groups compared it seems likely that the fatality rate of patients with cancer of the liver with cirrhosis is higher than, or at least just as high as that of patients with cancer of the liver without cirrhosis. Therefore if there is any bias in the comparison made in Table 1, it would create a spurious negative correlation between cirrhosis and ulcer. In other words had a positive correlation been found it would have been trustworthy. However the P value found is only about 0.10 and therefore no definite conclusion can be drawn.

As the next step in the analysis we compared the occurrence of peptic ulcer in the three groups of cirrhosis of the liver (Table 2).

TABLE 2

Incidence of Peptic Ulcer in Cases of Incipient, Moderate and Severe Laennec's Cirrhosis

	Peptic ulcer		
	+	—	Σ
Incipient cirrhosis	11	76	87
Moderate cirrhosis	14	51	65
Severe cirrhosis	22	45	67
	47	172	219

$\chi^2 = 9.15$ d.f. = 2 $0.02 > P > 0.01$

Table 2 shows a probably significant difference between the three groups compared. Peptic ulcer occurred most often among the cases with severe cirrhosis and least often among those with incipient cirrhosis. Now, cases with severe cirrhosis may be assumed to have a

higher death rate than those with moderate or mild cirrhosis The comparison made in Table 2 is therefore biased according to *Berkson* This bias would result in a spurious negative association between peptic ulcer and severe cirrhosis The positive correlation found must therefore be accepted as true as it exists in spite of the bias of this comparison This finding of a higher incidence of peptic ulcer with increasing grade of cirrhosis strongly suggests a causal connection of some sort between the two diseases

Finally we grouped the cases of hepatic cirrhosis according to age and sex and studied the subgroups so constructed (Table 3)

TABLE 3

Incidence of Peptic Ulcer in Cases of Incipient Moderate and Severe Laennec's Cirrhosis in Patients of Different Age and Sex

Age	Incip cirrhosis Peptic ulcer				Moderate cirrhosis Peptic ulcer				Severe cirrhosis Peptic ulcer			
	+		−		+		−		+		−	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
≦ 49	1	0	1	0	0	0	3	0	1	0	5	2
50–59	3	0	8	1	2	1	8	1	5	2	10	2
60–69	0	0	10	5	1	0	8	2	1	3	5	3
70–79	0	0	9	15	4	4	9	7	4	4	6	5
≧ 80	2	5	12	15	1	1	6	7	0	2	1	6
Σ	6	5	40	36	8	6	34	17	11	11	27	18

As seen in this table there were more young individuals among the cases graded as severe cirrhosis than among those graded as moderate and incipient This, no doubt, reflects the poorer life expectancy in the severe group Further, men predominated in all groups especially among the cases graded as moderate This predominance disappears, however, in the oldest age groups As shown by Table 3 in all the three groups of cirrhosis the cases of peptic ulcer seem to be rather evenly distributed among the different sexes and ages

DISCUSSION

In the present series of autopsy cases peptic ulcer in stomach and/or duodenum was found to occur more often in cases with than in those without Laennec's cirrhosis This finding was not accepted as proof of an association of the two diseases in the living population Further, though the incidence of peptic ulcer among cases of cancer of the liver with cirrhosis was found to be somewhat higher than among cases without cirrhosis, this difference was not significant and had therefore to be rejected Finally, however, peptic ulcer was found to occur significantly more often with increasing grade of cirrhosis This find-

ing was regarded as a strong indication of a causal connection between the two diseases

In grading the cases of cirrhosis according to the degree of fibrosis it was assumed that this degree gives an indication of the mortality risk It has long been known that fibrosis is responsible for the distortion of the circulation with resulting elevation of portal vein pressure met in cirrhosis (*Himsworth* 1950) Admittedly increased portal vein pressure is the cause of death only in a minority of cases many patients die either directly of liver failure or of impaired liver function complicated with infections However the recent study of *Willoughby David Smith Fruin & Baker* (1964) has shown clearly that impairment of the liver function generally parallels the elevation of the portal vein pressure which as pointed out before is chiefly caused by intrahepatic fibrosis We therefore felt to have good reasons for using the degree of fibrosis as an indication of the mortality risk We found further support for this when the average age of the patients turned out to decrease progressively with increasing grade of fibrosis This finding we interpreted as reflecting the higher death risks of the higher graded cases

Returning to the comparisons made it may be objected that in the two comparisons the results of which were accepted the only selection bias taken into account was that due to competing fatality rates *i e* due to selection by death However autopsy series are selected not only *by death but* also after death—some cases are autopsied others are not— *and before death* some patients are admitted to hospital others are not Selection after death may be ignored here as the autopsy rate *in this* hospital is almost 100 per cent Regarding selection before death it should be remembered that the comparisons accepted were comparisons *within a certain disease group* between subgroups with disease of varying severity In communities where hospital treatment is *free* as *in* Sweden the most important factor deciding whether or not a [illegible]

[illegible] cases are more often admitted to hospital and they also have a higher *death rate* The results of the comparisons accepted were found *to* be *valid* in spite of this kind of selection

The conclusion of this study therefore is that an association exists between hepatic *cirrhosis* and peptic ulcer This conclusion is in accordance with the results of animal experiments (*Bergman & van der Linden* 1965) The *reason for* this association is still unclear Venous congestion and malnutrition resulting in [illegible]

tric [illegible]

that [illegible]

tric [illegible]

SUMMARY

The occurrence of peptic ulcer in patients with Laennec's cirrhosis is studied in an autopsy series In this study special attention was focused on sources of error in such investigations From comparisons of sub groups with different chances of being drawn into the studied sample it is concluded that an association exists between hepatic cirrhosis and peptic ulcer in the living population

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AN ANALYSIS OF THE CELL POPULATION IN THE SPLEENS OF YOUNG MICE CHIMERAS

By

PETER EBBESEN and EMMERIK JENSEN

Received 14 iv 65

The finding by *Simonsen et al* (1) of enlargement of the spleens of ten days old mice injected at bitrh with homologous spleen cells raises the question whether this enlragement is due to colonization of the spleen with homologous cells or to proliferation of host cells

Davies & Doak (2) have examined the distribution of cells in a suspension of spleens from such animals They used the chromosome-marker technique by which cells in division can be identified With this technique they found, with a single exception, no donor cells in the suspension They examined spleens from animals four to ten days of age and a few that were 6 months old

In the present paper we have tried to analyse the cell population from young chimera mice injected when new-born with homologous spleen cells The spleens were examined with a cytotoxic antiserum technique originally developed by *Gorer & O Gorman* (3) The spleens examined were taken on day 2, 3, 4, 7, 8, 10 and 12 We have chosen this technique in the hops of being able to determine the composition of the total cell population at the given time and not, as is the case when the chromosome-marker technique is used, only those cells that divide at the moment of examination

MATERIALS AND METHODS

Mice

Inbred new born mice of the strains C_3H and ST/A mice have been used These strains have been shown by *Simonsen et al* (4) to be mutually strongly antigenic with a high spleen index They have been shown to differ at the H 2 locus C_3H being H 2k and ST/A to be H 2b *Gorer* (5)

Injection of the New born Mice

Within 24 hours after birth the mice were injected intraperitoneally with 5 10^6 adult spleen cells

Preparation of the Cell Suspension

The spleen from a 6 month-old mouse was removed and homogenized in a Potter Elvehjem homogenizer containing 1 ml of Tyrode s salt solution and strained through a fine gauge mesh to remove cell clumps

Immune Sera

Homologous antisera were prepared in adult C3H and ST/A mice by repeated intraperitoneal injections of a cell suspension containing one third of an adult spleen. The injection was repeated after one month and thereafter twice at a weekly interval. Serum was obtained 10 days after the last injection.

Complement

Freshly prepared pooled guinea pig serum obtained at the same day by heart puncture was used.

Cytotoxic Technique

The modification of Gorer and O'Gorman's original technique as given by *Boyse* (6) was used.

The spleens from the injected animals were very cautiously cut longitudinally and placed in a few drops of normal undiluted isologous mouse serum. After removing the spleens the cell suspension was mixed by a pipette. One drop of the cell suspension was mixed with one drop of antiserum diluted 1/5 with Tyrode's solution. This dilution was used in order to minimize the known anticomplementary effect of mouse serum. The mixture was placed on a clean glass slide with fat removed and rocked gently to mix. After incubation for 15 minutes by 37° C in a humid chamber one drop of guinea pig serum was added and incubated for another 40 minutes. After adding one drop of a freshly prepared Trypane blue solution (1 part 4.5 per cent saline added to 4 parts stock Trypane blue of 1.25 per mille) the cell suspension was covered with a cover slip and examined under the microscope. Killed cells take up the dye and the relative distribution of killed and living cells can be directly counted.

Specificity of Sera

The immune sera used were tested for specificity against spleen cells from the homologous strain before use in the experiment.

TABLE 1

Target cells	Serum	Stained/unstained cells
ST/A	C3H anti ST/A	96/4
	ST/A anti C3H	3/97
C3H	C3H anti ST/A	2/98
	ST/A anti C3H	96/4

This was repeated with serum diluted 1/25 to ensure a sufficient potency of the antisera used. The results are shown in Tables 1 and 2.

TABLE 2

Target cells	Serum	Stained/unstained cells
ST/A	C3H anti ST/A	98/2
	ST/A anti C3H	3/97
C3H	C3H anti ST/A	2/98
	ST/A anti C3H	97/3

The sera used thus show the necessary specificity and strength.

The non specific cell death or survival were in all cases less than 5 per cent.

TABLE 3

Age days	Donor strain	Recip strain	Sera	No of stained cells	No of unstained cells	% stained cells	Corrected % of stained cells	% homologous cells
3	C3H	ST	anti ST	79	21	79	85	15
			anti C3H	24	76	24	17	17
3	C3H	ST	anti ST	76	24	76	81	19
			anti C3H	20	80	20	13	13
3	Control	ST	anti ST	92	8	92		
			anti C3H	9	91	9		
3	ST	C3H	anti ST	22	78	22	19	19
			anti C3H	84	16	84	85	15
3	ST	C3H	anti ST	20	80	20	17	17
			anti C3H	72	28	72	74	26
3	Control	C3H	anti ST	5	95	5		
			anti C3H	97	3	97		
4	C3H	ST	anti ST	43	7	86	47	6
			anti C3H	11	86	11	8	8
4	C3H	ST	anti ST	43	7	86	47	6
			anti C3H	7	37	16	5	11
4	Control	ST	anti ST	65	3	84		
			anti C3H	4	70	5		
6 mth	Control	C3H	anti ST	1	100	1		
			anti C3H	100	3	97		
7	C3H	ST	anti ST	67	8	89	69	8
			anti C3H	11	89	11	7	7
7	Control	ST	anti ST	51	2	96		
			anti C3H	3	55	5		
6 mth	Control	C3H	anti ST	2	50	4		
			anti C3H	52	2	96		

RESULTS

The examination of the spleen from the two day old animals was difficult to carry through The number of cells harvested from the extremely small spleens was—by the careful manipulation specific cell death too low to allow an adequate counting The results are therefore not listed in tables As far as one can judge however, they [illegible] at a considerable number of donor cells in the suspensions

The results of the examinations of the spleens from the mice aged 3 4 7 10 and 12 days are listed in Tables 3 and 4

As the cytotoxic effect of an specific antiserum is less than 100 per cent the following computation was done in an attempt to reach the actual distribution of C3H and ST/A cells in a suspension

TABLE 4

Age in days	Donor strain	Recip strain	Sera	No of stained cells	No of unstained cells	% stained cells	Corrected No of stained cells	% homologous cells
8	ST	C3H	anti ST	4	50	8		not calculated
			anti C3H	40	5	84		
8	Control	C3H	anti ST	2	50	4		
			anti C3H	40	2	95		
10	C3H	ST	anti ST	35	3	92		
			anti C3H	1	35	3		
10	C3H	ST	anti ST	35	7	82		
			anti C3H	1	35	3		
10	Control	ST	anti ST	50	2	96		
			anti C3H	0	50	0		
10	ST	C3H	anti ST	2	35	5		
			anti C3H	35	4	90		
10	ST	C3H	anti ST	1	35	3		
			anti C3H	20	2	91		
10	Control	C3H	anti ST	2	80	2		
			anti C3H	70	2	97		
12	C3H	ST	anti ST	94	6	94		
			anti C3H	6	94	6		
12	C3H	ST	anti ST	95	5	95		
			anti C3H	7	93	7		
12	Control	ST	anti ST	96	4	96		
			anti C3H	4	96	4		
12	ST	C3H	anti ST	6	94	6		
			anti C3H	97	3	97		
12	ST	C3H	anti ST	7	93	7		
			anti C3H	94	6	94		
12	Control	C3H	anti ST	6	94	6		
			anti C3H	96	4	96		

The number of killed cells of a certain type equals the total number of this cell type (x) multiplied by the factor (k) which indicates the proportion of these cells which are killed by specific antiserum. This factor is determined in the control experiment.

The total sum of killed cells of both cell types equals the total number of stained cells in the suspension. This sum is called S.

Formula 1 $S = X \times k + Y \times f$

S the number of stained cells
X the predominant cell in the suspension (for example ST/A cells in a ST/A spleen)
k the proportion of the X cells stained in the control suspension using the same antisera

X and f the same symbols for the other cell type present in the suspension The total number of living and dead cells in a suspension (Z) equals the number of C_3H and ST/A cells

Formula 2 $Z = X + Y$
Z the total number of cells counted

Formula 3 $Y = \frac{S \quad Z \times f}{h - f}$

The computed number of the dominant cell for example ST/A cells in a ST/A spleen shows the corrected number of *stained* cells (column 8) directly when an anti ST A serum is used whereas it shows the corrected number of *unstained* cells when an anti C_3H serum is used In this case the corrected number of stained cells shown in column 8 is arrived at by substraction from the total number of cells in the suspension

The corrected number of stained cells directly shows the number of donor cells when a serum is used directly against the donor If a serum directed against the recipient is used the number of host cells is shown in column 8 as the corrected number of the most frequent type of cell among the stained cells By substraction from the total number of cells the number of donor cells is arrived at Their number is shown in column 9

The proportion of donor cells in the spleen suspensions The percentage of donor cells thus a[illegible]
19 in the 3 day old C_3H mice
3 per cent donor cells was fo[illegible]
old ST/A mice

Concerning the 8 10 and 12 days old mice the proportion of stained cells in the experimental animals was so close to that of the controls that no significant difference can be shown

(In a small number of the 10 day old mice the spleen index was determined (the relative spleen weight compared to the average relative spleen weight among the control animals from the same litter)

3 out of 4 animals showed a definite increased spleen index indicating spleen enlargement In these animals no donor cells were found in the spleen suspensions

In contrast to the findings by *Davies & Doak* we have found a number of donor cells in the spleen from young chimera mice within the first week after injection of the homologous cells This is probably due to the cytotoxic technique used in the present paper This technique reflects the total cell population at a given time whereas the cell marker technique can only show cells in division at that moment

The steep decrease in the number of donor cells found in the present study during the first ten days after transplantation indicates an accordance with findings by *Davies & Doak* that the donor cells are rapidly eliminated in the host organism

The spleen enlargement on the tenth day after injection of homologous cells as shown by *Simonsen & Jensen* (and confirmed in this paper) must thus be due to a proliferation of host cells

SUMMARY

The spleen enlargement found by Simonsen in 10-day-old chimera mice injected at birth with homologous cells has previously been taken as a result of proliferation of the injected cells Using the chromosome marker technique Davies & Doak found indications that this enlargement was due to proliferation of host cells Using a cytotoxic antibody technique—which in contrast to the chromosome marker technique shows not only the distribution of the cells in division but of the total cell population at a given moment—is has been shown in the present study that the injeceted cells can be found for a few days and then are eliminated Thus the spleen enlargement must be due to proliferation of host cells

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LOCAL LABELLING OF LYMPH NODES WITH TRITIATED THYMIDINE

By

STURE LIDÉN and JUHANI LINNA

Received 27 iv 65

Among the lymph nodes the superficial ones have a special significance because of their function as recipients of lymph from the body surface By means of this lymph, different substances entering the body via the skin can be transported to the regional lymph nodes Cutaneous application of the contact sensitizing agent 2,4 dinitrochlorobenzene has been shown to produce certain morphological alterations in the regional lymph nodes of guinea pigs (*Macher* 1962) These lymph nodes are also necessary for the induction of contact sensitivity in the guinea pig (*Frey & Wenk* 1956) It is unclear, however why they are necessary for this process It seems possible that there is an export of cells from the regional lymph nodes to other parts of the body, and that this dissemination of cells gives rise to the universal sensitivity that results from local application of a contact sensitizer

To facilitate a study of the fate of the lymph node cells a method for local labelling of these is desirable The deoxyribonucleic acid (DNA) can be specifically labelled (*Reichard & Estborn* 1951) with tritiated thymidine later referred to as H^3-TdR (H^3 Thymine deoxyRibose) This compound is much in current use as a label in studies of cellular kinetics For basic principles and references on this subject see *Hughes, Bond Brecher Cronkite Quastler & Sherman* (1958) and for a summary of recent modifying views *Bertalanffy* (1964) According to *Hudack & McMaster* (1932) intradermal injections can be regarded as intralymphatic because of the very fine mesh of the lymphatic networks which makes it impossible to carry out an intradermal injection without rupturing the lymph capillaries The first cells with a high mitotic activity which meet the lymph from the skin are the cells in the lymph nodes regional to the injection H^3 TdR transported with the lymph could be expected to be utilized in the synthesis of DNA by the lymph node cells if the passage through the node were not too rapid The uptake of injected thymidine seems to start immediately it comes in contact with DNA synthesizing cells (*Potter* 1959 *Rubini Cronkite,*

The work was supported by the Swedish Medical Research Council

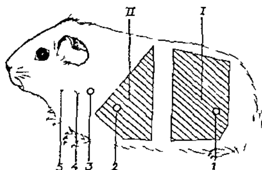


Fig 1

Side view of guinea pig Intradermal injections with H^3 TdR were performed either in area I or in area II Area I drains to the inguinal lymph node station (1) and area II to the axillary lymph node station (2) The localization of the superficial cervical lymph node (3), the deep caudal cervical (4) and the deep cranial cervical lymph node (5) is indicated

Bond & Fliedner 1960) It was therefore assumed that a certain degree of local labelling would follow intradermal injection of H^3-TdR This assumption was confirmed during the course of this investigation by *Miller III* (1964) By means of autoradiography he found a local H^3-TdR labelling of the popliteal lymph nodes of rats, injected into the hind footpads at different times before killing

The aim of this experiment was to investigate whether intradermal injection into a certain skin area with H^3-TdR would give a labelling more pronounced in the lymph node station regional to the injection than in other lymph nodes, and to determine the rate of disappearance of the local label

AUTORADIOGRAPHIC STUDIES

Experiment I

This experiment was set up to ascertain the possibilities of carrying out a partly selective H^3 TdR labelling of the lymph nodes regional to intradermal injections of the label, and of its localization and persistence in the lymph nodes

Material and Methods

Animals

14 male (*Cowdry* 1953) albino guinea pigs weighing 220–340 g at the time of labelling were used The animals were of a mixed stock obtained from the same breeder, and kept on a standard diet of roots and hay After injection of H^3 TdR the animals were caged separately

Labelling

The fur on the injection areas was removed with an electric clipper (Type Komet Veb Elektrogeratewerk Germany) on the day before the injections and the skin shaved with an electric shaver (Braun Germany) ½ 2 hours before the injections These were all made between 7^{30} and 9^{30} a m (*Messier & Leblond* 1960) The skin areas draining to the inguinal (= subiliac) and axillary lymph nodes were determined according to *Keller* (1937) Lines marking the area to be injected were drawn

½ 1 cm in a central direction from these borders (Fig 1) No injections were made into the skin of the legs

Experimental Plan

The animals were killed by a blow on the head at the time intervals described

days after labelling

For more elaborate autoradiographic counting the inguinal lymph node stations

of lymph nodes

Histologic and Autoradiographic Technique

The lymph nodes were placed in 4 per cent carbonate buffered formalin for subsequent ethanol dehydration and xylene clearing according to the usual methods

(*Everett & Simmons* 1953) Kodak autoradiographic stripping film type AR 10 was used and the technical procedure was largely that described by *Pelc* (1956)

Examination of Autoradiograms

The autoradiograms were

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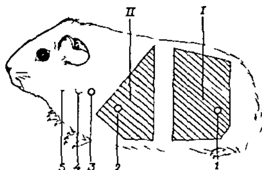


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½ 1 cm in a central direction from these borders (Fig 1) No injections were made

Bioresearch, Inc, N Y, USA) was injected intradermally animal either in the area

Experimental Plan

The animals were killed by a blow on the head at the time intervals described below and the lymph nodes were dissected out For autoradiographic screening,

mals the regional and contralateral axillary lymph nodes were taken and also some of the homolateral neck lymph nodes (the superficial cervical, the deep cranial cervical and sometimes also the deep caudal cervical lymph nodes, Fig 1) Of the latter group of animals 2 were killed 24 hours after, and the remaining 4 animals

as control lymph nodes

Histologic and Autoradiographic Technique

The lymph nodes were placed in 4 per cent carbonate buffered formalin for sub

containing silica gel They were developed in Kodak 19 b for 5 minutes and rinsed in distilled water before fixing in Kodak acid fixer for 10 minutes All three steps were carried out at exactly +18° C The sections were stained with haemalum according to *Mayer* (*Romeis* 1948)

Examination of Autoradiograms

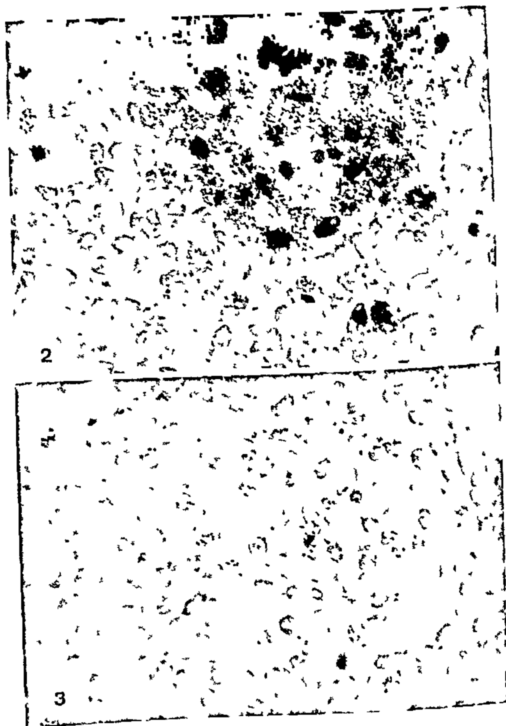
The autoradiograms were observed under about 1,600 × magnification using an oil immersion lens and an optic micrometer in the ocular

In the *screening* of the *autoradiograms*, the number of grains above the nuclei in the pale staining parts of the centre-containing secondary nodules (see Figs 2 and 3) was estimated

by *Rin*

(*Flemm*

In the *detailed autoradiographic* counting of the



Figs 2 3

Fig 2 Autoradiogram showing a centre containing secondary nodule of an inguinal lymph node 24 hours after regional intradermal injection of 25 μc H^3 TdR × 540

Fig 3 Autoradiogram showing a centre containing secondary nodule of an inguinal lymph node contralateral to the lymph node from which Fig 2 is taken × 540

the control lymph *node stations, no differences* in the degree of labelling were encountered Therefore no selection was necessary in this latter case

The background grains in the film not overlying the sections were about 15 per 1 000 μ^2 The cells in sections are small as compared with the cells in smears, furthermore, in 5 μ *thick sections, a certain overlapping of cells occurs and so the* number of cells per 1 000 μ^2 was in the order of 25 Thus a grain number per nucleus of 5 or more was considered to indicate a high probability of true labelling especially as the background grains are fewer in number above the sections than in other parts of the film (*Tonna & Cronkite 1958*) *When calculating the mean grain* count cells with more than 50 grains were arbitrarily regarded as having 60 grains

Results

The screening of the autoradiograms revealed certain distinct features *In some, but not all, of the lymph nodes from the* station regional to the labelling, the nodular cells were heavily labelled 24 hours after the H³-TdR injection (Fig 2) The corresponding cells of the control lymph nodes showed a much weaker labelling (*Fig 3*) *Here the labelling* was of the same intensity in all the different nodes of the station These differences in grain number between regional and control lymph nodes were less pronounced in the animals killed 2 and 3 days after labelling, than in the animals killed 24 hours after labelling Above all, now there were no heavily labelled nodular cells in the lymph nodes regional to the injection In a few lymph nodes, evaluation was difficult because of the sparsity of centre-containing secondary nodules On the other hand, the non-nodular cells of the regional lymph nodes showed many heavily labelled cells even 3 days after labelling In the control lymph nodes no heavily labelled nodular cells were found, and of 1,000 labelled non nodular cells *screened, only 3 had more than 50 grains*

TABLE 1

Animal	Nodular cells		Non nodular cells	
	Regional lnn	Control lnn	Regional lnn	Control lnn
	Percentage of labelled cells			
159	78 8	42 6	4 4	5 0
160	71 6	32 0	4 5	4 8
	Total grain count over 1 000 cells			
159	26 203	4 832	946	664
160	18 716	3 990	1 214	721
	Mean grain count over labelled cells			
159	33	9	18	9
160	26	10	24	11

Throughout this part of the experiment there was a distinct difference between the lymph nodes regional to the labelling and all the control lymph nodes Thus there was no sign that the marker crossed

the borders between the different lymph drainage areas via the lymphatics neither when the inguinal nor when the axillary areas were injected according to the principles followed here

The results of the detailed counting of the regional and control lymph nodes from 2 animals are shown in Table 1 All the values obtained are consistently higher for the regional lymph nodes, except the percentages of labelled cells which show no certain differences in the non nodular cell groups The total grain count values are taken as an estimate of the specific activity of the cells in question The relation of the total count (nodular + non-nodular cells) of the regional to that of the control lymph nodes is in animal 159, 4 9 1, in animal 160, 4 2 1 The mean grain counts of the labelled cells show less pronounced differences between regional and control lymph nodes Heavily labelled cells with more than 50 grains per nucleus, however, were found mainly in the regional lymph nodes Of the 400 labelled cells screened in the non nodular tissue and of the 2,000 cells counted in the nodular tissue of *the control nodes, only 2 cells showed a grain count exceeding 50*

LIQUID SCINTILLATION STUDIES

Experiment II

Liquid scintillation counting of tritum activity is more accurate than autoradiographic counting in quantitative work The greater accuracy of the former method is due to its higher efficiency The efficiency of autoradiography with H^3-TdR as marker is in the order of 0 5 per cent (*Kisieleski, Baserga & Vaupotic* 1963) Liquid scintillation counting with *Bray*'s solution and 10 per cent tritiated water has an efficiency of 11 7 per cent (*Bray* 1960) Because of the presence of trichloroacetic acid (TCA) causing quenching in the counting system, the efficiency of our countings is reduced to about 8 per cent

The following experiment was performed in order to obtain a clearer view of the degree of the local lymph node labelling and of the relationship between time and tritium activity found in experiment I

Material and Methods

Animals

10 animals of the same kind as those in experiment I were used Their weights varied between 230 and 290 g 8 animals were injected with H^3 TdR and 2 animals served as controls receiving no isotope

Labelling

The animals were labelled by injecting 25 μc H^3 TdR into the left inguinal area in the same way as in experiment I

Experimental Plan

The inguinal lymph node stations were collected from both sides of the 8 animals injected one pair at a time 24 hours 3 5 and 7 days previously These lymph node stations were also taken from the 2 non injected control animals

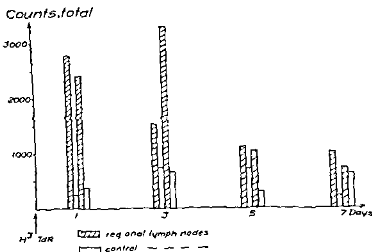


Fig 4

Total liquid scintillation counts per 10 minutes of inguinal lymph node stations regional to intradermal injection of 25 μc H³-TdR and of the contralateral inguinal (control) lymph node stations 2 animals at each time

Isolation of DNA and Liquid Scintillation Technique

DNA was extracted by a modified *Schneider* procedure (*Schneider* 1945) in the following way The lymph nodes were homogenized with a refrigerated *Bühler* homogenizer for approximately 1 minute in 5 ml of 5 per cent trichloroacetic acid (TCA) The homogenate was then centrifuged at +4° C at about 2 000 g for 10

The radioactivity was measured in a liquid
314 ex at +2° C
per cent water pha
isolated in the same
animals provided
(*Baillie* 1960) was
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Results

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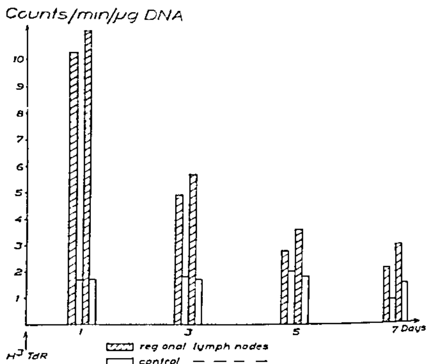


Fig 5
Specific activities (counts/minute/µg DNA) of the same lymph node stations as in Fig 4

usually high DNA content of these nodes In the other samples, the amount of DNA varied from one lymph node station to the other, without correlation to either the injected or the non-injected side The total counts of the symmetrical contralateral, *i e* control lymph nodes, vary relatively more When the values of total counts are related to the total amount of DNA in the nodes, *i e* when the specific activities are calculated, the differences are largely eliminated (Fig 5)

Thus the determinations on chemical bases show a labelling of the regional lymph nodes exceeding that of the control nodes with a factor 7–12, 24 hours after labelling, as judged from the values of the total activity, and with a factor 6 7, as judged from the specific activities The small absolute variations in activity of the control nodes, compared with the rapid decrease in activity of the regional nodes are a striking feature both in Fig 4 and in Fig 5

DISCUSSION

The results show that it is possible, by intradermal injection, to obtain a labelling with H^3-TdR which is considerably stronger in the regional lymph node station, than in the neighbouring control lymph node stations With the H^3-TdR doses and the intervals between injection and killing chosen in this experiment, it is also evident that a certain "leakage" occurs beyond the regional lymph nodes The control

lymph nodes have been chosen to reveal a possible utilization of the lymphatic communicants between bordering skin areas draining to different lymph node stations as described by *Forbes* (1937–38) for human skin However, the question of whether or not there is a crossing over between the axillary and inguinal skin areas has not been investigated here No exact information concerning the occurrence of valves in the different lymph plexus of guinea pig skin has been found in the literature In any case, a flow direction against valves is considered to be a consequence of mechanical factors such as experimental high intralymphatic pressure and possibly massage (*Hudack & McMaster* 1932) On the other hand, in the absence of valves, as for example in the superficial dermal lymph capillary plexus of man (*Kampmeier* 1928), even gravitation could be a factor of importance for the direction of the lymph flow Thus lymph passage over the vertical border between axillary and inguinal lymph drainage areas does not seem more probable than passage over the ventral midline It is therefore unlikely a) that the passage of label between homolateral axillary and inguinal lymph nodes is higher than the passage to the contralateral symmetrical control nodes, and b) that the general labelling is higher than the labelling of these control lymph nodes

It is possible that the border between the skin areas draining to the neck lymph nodes and to the axillary lymph nodes can vary to a certain degree (*Keller* 1937) In order to ascertain whether the cranial border chosen for the injections was safe with regard to the selective labelling of the axillary lymph nodes, autoradiograms from the neck lymph nodes were also screened In the animals investigated, however, no signs of selective labelling were found in the neck lymph nodes

Of the possible routes of injection, only the intradermal and subcutaneous came into consideration According to the earlier mentioned work of *Hudack & McMaster* (1932), intradermal injections can be regarded as being intralymphatic The significance of the skin blood capillaries as pathways for the label going beside the lymph nodes is diminished by their higher mechanical resistance, as compared with that of the lymph capillaries (*McMaster & Hudack* 1932) The trauma of injection is thus more likely to disrupt the lymphatics than the blood vessels Subcutaneous injections have given good general labelling with H^3 TdR (*Messier & Leblond* 1960) Because of these facts, the intradermal route was chosen as the one being most likely to direct as great a part as possible of the H^3-TdR to the regional lymph nodes Since guinea pig skin is very thin it is difficult completely to avoid accidental escape of the injected material into the subcutaneous tissue However, a well directed transport of the injected material can be obtained even without strict intradermal injection (*McMaster & Hudack* 1935)

The localization of the label to the centre containing secondary nodules is in accordance with the results obtained by general labelling (*Messier & Leblond* 1960, *Edwards & Klein* 1961) and also with the

formerly quoted local labelling experiment by *Miller* (1964) It also agrees with the fact that the lymph node sinus walls are incomplete wherever lymphatic growth is active (*Drinker & Wislocki* 1933), thus permitting free flow between the sinuses and the cells

The stability of the label in the control lymph nodes is in agreement with the findings by *Schooley, Bryant & Kelly* (1959) if the total lymph node cell population is considered, and also with the findings by *Mitchell, McDonald & Nossal* (1963)

There is a considerable difference between the labelling in the regional lymph nodes and in the control lymph nodes in the first few days after the H^3-TdR injection It must be stressed, however, that the amount of the injected H^3-TdR present in the regional lymph nodes even in the 24-hour-group is but a small fraction of the amount injected The resulting general labelling makes identification impossible of the individual cells from the regional lymph nodes However, heavily labelled cells occur mainly in the regional lymph nodes When such cells are found in other places it is probable, to a certain degree, that they have emigrated from the regional lymph nodes (*Miller* 1964) The presence of a selective attraction of the cells from the lymph node regional to the labelling, would be reflected in an increased number of labelled lymphoid cells in the area to which they are attracted

The possible radiation damage by the tritium isotopes must be kept in mind The low weight of the lymph node stations (10–20 mg wet weight as a rule) makes the relation between tissue mass and quantity of injected isotope, questionable from a radio-toxicological point of view (*Plaut* 1959, *Johnson & Cronkite* 1959, *Lisco Nishimura, Baserga & Kisieleski* 1961, *Baserga & Kisieleski* 1962, *Garder & Devik* 1963) The fact that not all lymph nodes of the regional lymph node station are labelled—probably because the whole skin area draining to these nodes was not injected—makes this relation even more unfavourable However, the quantity of H^3 TdR which is built into DNA is very small and the cells are probably exposed for only a very short time to the relatively large amount of isotope injected

Having due regard to the limitations of this method of labelling of the regional lymph node cells, it seems probable that it can be of value in studies concerning the fate of these cells It seems to suffer from fewer unphysiological traits as compared with a method used earlier in such experiments with transfusion of *in vitro* labelled cells

SUMMARY

The local labelling effect of tritiated thymidine, injected intradermally into skin areas draining to the inguinal or axillary lymph node stations, has been investigated by means of autoradiography and liquid scintillation counting of these lymph nodes

A certain degree of local labelling of the lymph nodes draining the

injection area was found, and this labelling was most readily detectable in the centre containing secondary nodules After 24 hours there was a difference in specific activity between the lymph nodes regional to the labelling injections and the control lymph nodes of about 6 1 This difference gradually decreased during the 7 day period of investigation Autoradiographic screening of the lymph nodes draining skin areas adjacent to the skin areas injected with the marker, showed the chosen borders for the injections to be safe with regard to possible, but unintended crossing over of the label via the skin lymphatics Although the labelling by no means is strictly localized to one lymph node station the usefulness of the method for studies of the fate of lymph node cells is pointed out

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ADDENDUM

After having prepared this manuscript we became aware of a pertinent experiment by *R A Prendergast* (*Cellular specificity in the homograft reaction* J Exp Med *119* 377 1964) He showed by means of autoradiography that repeated intradermal injections of small amounts of H^3 TdR into the ear of rabbits yielded a local labelling of the auricular lymph node without any significant general labelling

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ACID MUCOPOLYSACCHARIDES IN THE RASK NIELSEN TRANSPLANTABLE MOUSE MASTOCYTOMA

By

ROBERT BRUNISH and GUSTAV ASBOE HANSEN

Received 24 iii 65

The production of acid mucopolysaccharides by mast cells has been
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by mouse mast cells (*Marx & Spoller* 1961) The Dunn and the Furth transplantable mouse mastocytomas have been shown to produce heparin as well as other acid mucopolysaccharides (*Ringertz* 1960) There is evidence that mast cells are also responsible for the production of hyaluronic acid (*Asboe Hansen* 1950 and 1964) The cytochemistry and functions of mast cells vary not only between animal species but also between tissues and regions and even between neighbouring cells Normal human mast cells have not been shown to contain or release heparin and in human mastocytosis hyaluronic acid and chondroitin sulphuric acid were found in blood and urine, while no traces of heparin could be demonstrated (*Asboe Hansen & Clausen* 1964 a and b)

The present study was undertaken to investigate the Rask Nielsen transplantable mouse mastocytoma (*Rask Nielsen & Christensen* 1963) in regard to its mucopolysaccharide content The cells of this tumour resemble morphologically and histochemically the mast cells in tissues of normal mice (*Christensen et al* 1963)

MATERIAL AND METHODS

Tumour tissue was obtained from 4 female (*DBA/2 × CBA*) F_1 hybrids of the third transfer passage killed 10 months after subcutaneous inoculation. The tumour consisted of soft whitish masses similar to those observed by *Rask Nielsen & Christensen* (1963)

Samples of the
cent solution
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This work was supported by the USPHS grants NB 03998-03 and AM 06209-02
Our thanks are due to R Rask Nielsen PhD., University Institute of Biochemistry for supplying the tumour mice

For chemical analysis a major portion of the tumour was dried at 105° C for twenty four hours to determine the water content The dried residue was then employed for determination of hexuronic acid by the carbazole method (*Dische* 1947) and for determination of glucosamine and galactosamine by the column chromatography method of *Gardell* (1953) For the determination of hexuronic acid, 20 4 mg of dried tissue was homogenized in 0 05 N NaOH After two hours at room temperature the suspension was centrifuged at 3000 rpm and the opaque super natant fluid made up to 10 ml Analyses were made upon 1 ml aliquots

For the determination of hexosamines 25 mg samples of the dried tumour were homogenized in 4 ml of a 2 N HCl solution This suspension was hydrolysed at 117° C for 4 hours filtered and taken to dryness While insufficient tissue was available to control the destruction of hexosamine during hydrolysis this protocol used routinely and applied to skin, cartilage and other tissues results in minimal loss The residue was redissolved in 0 3 ml of 0 3 N HCl This solution was then applied to 400 mesh Dowex 50W × 8 columns as in the procedure of *Gardell* (1953) An additional 25 mg sample was homogenized in phosphate buffer pH 6 0 to which 0 5 mg of testicular hyaluronidase (LFO Helsingborg batch 15734 3000 VRE mg) or 0 5 mg of bacterial hyaluronidase (ORGANON batch H 590, 4322 TRU/mg) was added This large amount of enzyme was taken in order to overcome any inhibitory effect of heparin The sample was dialysed at 37° C against buffer for four hours after which another 0 5 mg of hyaluronidase was added and the dialysis continued for an additional twenty hours The hyaluronidase treated sample was then digested with hydrochloric acid and chromatographed under conditions identical with those of the untreated samples

A portion of 103 mg of the mastocytoma was stored in frozen state for 24 hours The following day it was homogenized in a micro glass tube and pestle with 0 1 ml

ith an additional 0 02 ml

for 20 minutes at 15 000

clear yellow supernatant

was obtained 0 005 ml was taken for electrophoresis on cellulose acetate strips The remainder was incubated with 0 04 mg hyaluronidase for 2 hours in a micro dialysis chamber against phosphate buffer After two hours an additional 0 04 mg of hyal uronidase was added and the incubation continued overnight Control samples, incubated without enzyme, were employed in order to determine whether any of the native acid mucopolysaccharide was dialysable under the conditions employed The following day the hyaluronidase treated sample was electrophoresed with the un treated sample The 0 5 M lithium acetate buffer and Alcian blue staining for acid mucopolysaccharide described by *Foster & Pearce* (1961) were employed Additional strips were stained for protein with 0 5 per cent amido black in 7 5 per cent acetic acid

RESULTS

Histology The tumour tissue consisted of densely packed tissue mast cells with toluidine blue metachromatic cytoplasmic granules (Fig 1) The granularity was varying In some areas all cells were loaded with granules, while in some other areas the predominant cells were less intensely granulated, but still clearly metachromatic The cytoplasm of both types of cells was stainable with Alcian blue pH 2 5 and 0 4 The cells did not differ essentially from normal tissue mast cells The cen tral nuclei were round, the chromatin structure relatively loose, and the cytoplasm was relatively abundant Besides the small vessels cross ing the tumour and occasional fibroblasts no other cells could be de tected The overwhelming mass of the tumour consisted of mast cells, while other constituents amounted to a few per cent

Chemistry Table 1 shows the chemical analyses for the mastocytoma samples, before and following hyaluronidase treatment

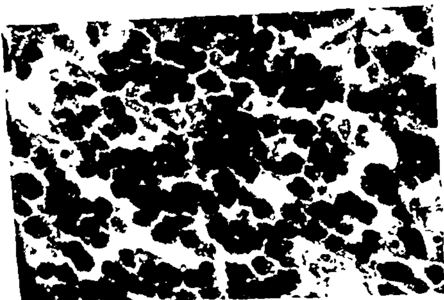


Fig 1

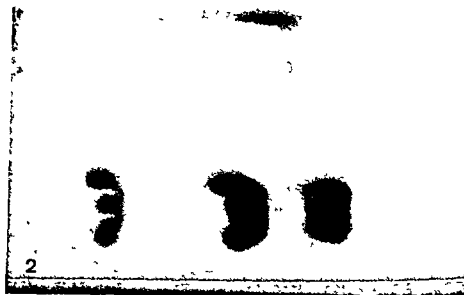
Densely packed well granulated mast cells of the mastocytoma Staining Toluidine blue, aqueous solution ½ per cent Magn approx 1000 ×

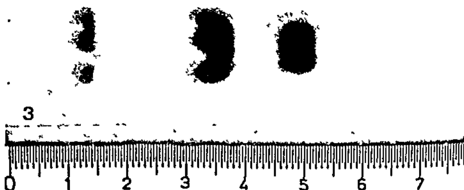
TABLE 1

Chemical Composition of Mastocytoma

Per cent water	76 8
	g per 100 g dry weight
Total hexosamine (as the hydrochloride)	1 10
Glucosamine	0 89
Testicular hyaluronidase resistant	0 43
Testicular hyaluronidase sensitive	0 46
Galactosamine	0 21
Testicular hyaluronidase resistant	0 07
Testicular hyaluronidase sensitive	0 14
Hexuronic acid	1 28

Figs 2 and 3 show the electrophoretic patterns obtained for the mastocytoma with and without hyaluronidase treatment For reference, a mixture of vitreous body hyaluronic acid (*Brunish et al* 1954), chondroitin sulphate of bovine nasal septum (SIGMA), and heparin (LEO) has been placed on the strip and run at the same time The heparin is partially resolved into a major fast moving component and a more faint slower component The mastocytoma shows bands which have the mobility of the slower heparin component, two in the chondroitin sulphate area another between chondroitin sulphate and hyaluronic acid and also a band which does not move from the point of application In certain runs in which the concentration of acid mucopolysaccharide was greater, the faster moving components were not clearly resolved, but an additional band was seen to have the mobility of hyaluronic acid





Figs 2 3

Fig 2 Electrophoretic patterns of acid mucopolysaccharides extracted from the mastocytoma (above) and of a mixture of hyaluronic acid chondroitin sulphate and heparin (below) Cellulose acetate strips 5 × 36 cm were employed, with 0 5 M lithium acetate Duration of the run was 5 hours at 100 volts Samples were placed at position indicated zero and have migrated to the right Stained with Alcian blue in 50 per cent ethanol buffered at pH 3

Fig 3 Identical to Fig 2 except that the acid mucopolysaccharides from the mastocytoma have been digested with testicular hyaluronidase and dialysed prior to electrophoresis



Fig 4

Tracings of the electrophoretic patterns of the acid mucopolysaccharides extracted from the mastocytoma The upper curve is untreated while the lower curve is that for the testicular hyaluronidase digested sample

Protein was seen only at the origin Upon treatment with testicular hyaluronidase (Fig 3) only the fastest moving component remains although a small amount of Alcian blue staining material may still be seen at the origin Treatment with bacterial hyaluronidase removed the hyaluronic acid band and greatly reduced that band lying intermediate between chondroitin sulphate and hyaluronic acid No loss of acid mucopolysaccharide due to dialysis was found when samples were incubated without enzyme Fig 4 represents the same patterns as analyzed by the Joyce Chromoscan the upper curve being that of the untreated sample and the bottom curve that for the testicular hyaluronidase treated sample In comparing quantitatively the two curves however it must be taken into consideration that the one sample has been diluted by enzyme solution

DISCUSSION

Considering the overwhelming accumulation of mast cells of varying granularity in the tumour it must be considered highly probable that the chemical analysis reflects the chemical composition of the mast cells It can not be excluded however that the intensely stained cells and the lighter cells have a different pattern and content of acid mucopolysaccharides The complexity of the electrophoretic pattern plus the data concerning the glucosamine galactosamine content before and after hyaluronidase treatment suggests that mouse mast cells contain other acid mucopolysaccharides in addition to heparin It may be speculated however that some components originate from connective tissue matrix or from blood vessels In the analyses of *Ringertz* (1960) on the Dunn and Furth mast cell tumours a complex pattern of acid mucopolysaccharides was seen following chromatography on ECTEOLA cellulose Ringertz obtained the same chromatogram when he studied ascites sublines rather than the total tumour and hence he was of the opinion that all the components had their origin in the mast cell The result of our histological investigation would also support such conclusion since relatively minor amounts of toluidine blue metachromatic material was seen in the intercellular substance

The chemical analyses obtained on the present tumour are also in keeping with those of *Ringertz* (1960) The present tumour had 19 1 per cent of its hexosamine as galactosamine while Ringertz found 24 per cent galactosamine in the Dunn mastocytoma and 20–40 per cent

in the Furth tumour All the hexosamine was calculated to be in the form of acid mucopolysaccharide since this value is close to that for hexuronic acid The maximum per cent of the acid mucopolysaccharide which could be heparin or heparin-precursor is relatively low *Silbert* (1963), studying the synthesis of heparin in cell-free preparations obtained from mouse mast cells, found that a substance, presumably a precursor of heparin, was formed which was not sulphated, but which was resistant to hyaluronidase If this observation also applies to mastocytoma, then the total amount of heparin plus heparin precursor must be measured by the amount of glucosamine which is present in polymer resistant to hyaluronidase This is 39 1 per cent of the total An additional 41 1 per cent contains glucosamine but is sensitive to testicular hyaluronidase and hence appears to be hyaluronic acid-like The remaining 19 1 per cent contain galactosamine, and of this one third is resistant to hyaluronidase in common with chondroitin sulphate B while two thirds are sensitive to hyaluronidase like chondroitin sulphate A and C

The electrophoretic patterns do not lend themselves easily to the above analysis There are several reasons for this

1 The intensity of Alcian blue staining need not be the same for each acid mucopolysaccharide
2 The mobilities observed may depend upon viscosity, molecular interactions, or other factors in addition to molecular charge However, the bands seen, except that at the origin, do not reflect mucopolysaccharide bound to varying amounts of protein since no protein was observed in the region of Alcian blue staining
3 The given conclusion is the simplest one consistent with the analyses It does not encompass additional, hitherto unknown, acid mucopolysaccharides, the presence of undersulphated molecules or misleading electrophoretic mobilities due to viscosity or particle interactions

SUMMARY

1 A Rask-Nielsen transplantable mouse mastocytoma showed densely packed tissue mast cells with largely two types of granularity
2 From this tumour at least four acid mucopolysaccharides were separated by electrophoresis Only the fastest component was resistant to testicular hyaluronidase, while bacterial enzyme attacked only tho e components with mobilities slower than chondroitin sulphate
3 Analyses for glucosamine and galactosamine content before and after treatment with testicular hyaluronidase suggest that 39 1 per cent of the acid mucopolysaccharide could be heparin or heparin precursor, while 41 8 per cent appeared to be hyaluronic acid like, and the remainder a mixture of chondroitin sulphates

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INFLUENCE OF NEONATAL THYMECTOMY ON THE LYMPHATIC SYSTEM AND ON ITS REACTION TO EXOGENOUS THYROXIN IN GUINEA-PIGS

By

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Received 19 x 64

Thymectomy of young rats, mice and rabbits causes a depletion of lymphocytes, apparent as a reduction in their output from the thoracic duct and in the number circulating in the blood, as well as reduced weight of the lymphatic tissue (*Reinhardt* 1945, *Nakamoto* 1957, *Schooley & Kelly* 1958, *Bierring* 1960, *Metcalf* 1960, and others) The earlier in life thymectomy is performed, the more pronounced becomes the shortage of lymphocytes and, if carried out in highly immature animals as *e g* newborn mice, rats or golden hamsters, the deficiency becomes extreme, with death as the end-result (*Miller* 1962, *Jancovic et al* 1962 *Sherman et al* 1963)

In guinea pigs thymectomized at a low age, a decreased percentage of lymphocytes in the blood has been reported by *Comsa* (1957), and a decreased output of thoracic duct lymphocytes by *Reinhardt & Yoffey* (1956) According to *Comsa*, thymectomized young guinea-pigs become cachectic and die within one month of operation This finding, however, is contradictory to other investigations, in which thymectomy of guinea-pigs had no influence on their growth or survival (*Park* 1917, *Schultze* 1933, *Klein* 1938, *Gyllensten* 1953, and others) The most extensive investigation of the lymphatic system of guinea pigs thymectomized at birth was made by *Gyllensten* (1953), who found transient lymphatic hyperplasia at the age of 14 days, not remaining at the age of 28 days If the thymus of the guinea pig is of the same importance for postnatal growth of the lymphatic system as the thymus of other rodents, this transient hyperplasia might be expected to be succeeded by a reduction in weight of the lymphatic tissue and a reduced lymphocyte content In the present investigation, guinea pigs thymectomized at birth were examined at the age of 50 days to determine whether such changes occurred In addition, it was investigated whether thyroxin treatment could stimulate cell proliferation in the spleen or lymph nodes of guinea-pigs thymectomized at birth, as demonstrated in guinea pigs with intact thymus (*Gyllensten* 1953, *Ernström & Gyllensten* 1959)

MATERIAL AND METHODS

In total 48 litters of newborn guinea pigs were used consisting of 130 males and females They were thymectomized or sham operated within 24 hours of birth. The operations were performed under local anaesthesia (0 5 per cent Xylocaine® Astra) using the technique of *Gyllensten* (1953) The animals were kept together with the respective mothers and suckled for at least the first month of life The mothers and their weanlings were fed on cabbage turnips carrots and vitamin pellets The experimental animals were divided into four groups

Group SC Sham operated at birth Control treated
Group ST Sham operated at birth Thyroxin treated
Group TC Thymectomized at birth Control treated
Group TT Thymectomized at birth Thyroxin treated

Thyroxin treatment and control treatment respectively were started at the age of 41 days Treatment consisted of subcutaneous injection of thyroxin (Thyroxin Hoffman La Roche & Co., 50 µg kg body weight) or saline into the dorsum of the animals every 3rd day (at 41 44 and 47 days of age) At the age of 50 days the animals were killed Thymus cervical extremital (scapular and inguinal) and mesenteric lymph nodes and the spleen were dissected out quantitatively and weighed The relative organ weights were calculated (mg/100 g body weight) The organs were prepared for histological examination by fixation in Carnoy s solution (alcohol chloroform and acetic acid) embedding in paraffin cutting in 5 µ thick section and staining in methyl green and pyronine at pH 4 8

The ... count cells) 500 c count ing th 1 mn

... and the mean percentage of the various cell types The mean ratios of lymphocytes/reticular cells and pyroninophilic cells/reticular cells were also calculated

In view of the suggested intimate relation between the thymus and the spleen (*Fichtelius* 1960) the latter organ was especially investigated The number of relevant cells per standard v... amount of red and w... sections from differ... paper to a... s procedure a relation ... The splenic specimens ... estigation were stained with Weigert s haematoxylin

From the percentage of each cell type and the number of cells ... volume and the ... relative splen... absolute amou... In this calcula... did not differ ... groups Moreover it must be emphasized that the amounts of the *different* cell types are not directly comparable since no correction was made for differences in size of the cells

RESULTS

Sham Operated Animals

At the age of 50 days, the relative thymic weight was much greater in female than in male guinea pigs ($p < 0.001$) On th...

of lymph nodes (Table 1) Nor was any significant sex difference observed in the cell population of the spleen and lymph nodes of the sham-operated animals

TABLE 1

Relative Weight (mg/100 g Body Weight) of Thymus Lymph Nodes and Spleen of Female and Male Controls Sham Operated at Birth Mean ± Standard Error Examination at the Age of 50 Days

	No of animals	Thymus	Lymph nodes				Spleen
			Cervical	Extremital	Mesenteric	Total mass	
Females	11	174 2 ± 10 7	80 5 ± 4 7	48 9 ± 1 7	131 2 ± 7 8	260 3 ± 9 1	137 7 ± 5 8
Males	18	111 4 ± 8 4	87 6 ± 6 9	49 3 ± 3 0	123 8 ± 6 3	256 7 ± 12 3	133 2 ± 8 4
p value of difference		< 0 001	–	–	–	–	–
Females and males	29		84 9 ± 5 6	49 2 ± 2 0	126 4 ± 4 9	258 0 ± 8 4	134 9 ± 4 4

Thymectomized Animals

The neonatally resected thymuses weighed 295 ± 16 and 284 ± 27 mg/100 g body weight in female and male guinea-pigs, respectively (mean ± standard error, no significant sex difference) At the age of 50 days, the guinea pigs thymectomized at birth did not differ from normal animals in appearance and growth (Table 2), and no sex difference in body weight was present Any signs of a wasting disease did not become manifest in some additional animals even if they were kept until they were 6 months old

TABLE 2

Body Weight (g) of the Experimental Animals at Birth before the Start of Thyroxin Treatment (41 Days of Age) and at the End of the Experiment (50 Days of Age) Mean ± Standard Error

	No of animals	Body weight		
		At birth	At the age of 41 days	At the age of 50 days
Sham operated control treated	29	85 6 ± 3 0	341 0 ± 12 9	388 8 ± 13 0
Sham operated, thyroxin treated	25	76 8 ± 2 8	320 0 ± 10 2	352 8 ± 11 7
Thymectomized control treated	37	83 1 ± 2 7	330 3 ± 14 0	368 9 ± 13 9
Thymectomized, thyroxin treated	24	86 6 ± 3 8	356 9 ± 15 8	376 7 ± 13 9

At the age of 50 days a significant reduction in weight of the lymphatic tissue but not of the spleen, was recorded in the thymectomized animals as compared to the sham operated controls The reduction was about the same in both sexes (Table 3)

TABLE 3

Reduction in Lymphatic Tissue (mg/100 g Body Weight) 50 Days after Thymectomy of newborn Guinea Pigs Mean of Difference between Thymectomized and Sham Operated Animals ± Standard Error

	df	Lymph nodes				Spleen
		Cervical	Extramural	Mesenteric	Total mass	
Females	28	−9 8 ± 6 4	−11 9 ± 2 7	−24 8 ± 9 1	−45 4 ± 12 6	+ 0 5 ± 8 5
p			< 0 001	< 0 05	< 0 01	–
Males	33	−16 4 ± 8 2	−10 1 ± 3 7	−31 1 ± 8 3	−55 0 ± 15 9	− 8 5 ± 10 2
p		< 0 05	< 0 05	< 0 001	< 0 01	
Females and males	63	−13 9 ± 6 4	−11 1 ± 2 5	−26 4 ± 6 1	−49 2 ± 10 8	− 3 1 ± 6 2
p		< 0 05	< 0 001	< 0 001	< 0 001	–

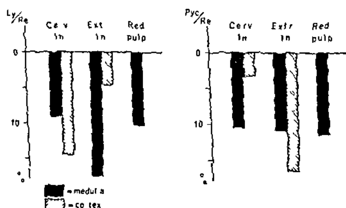


Fig 1

Effect of neonatal thymectomy on the ratios lymphocytes/reticular cells (left) and pyroninophilic cells/reticular cells (*right*) in different lymphatic organs at the age of 50 days Changes in per cent of values in sham operated controls

Furthermore neonatal thymectomy caused pronounced changes in the cell populations Thus *in all thymo lymphatic organs* examined the content of lymphocytes *and* pyroninophilic cells was decreased in comparison to the content of reticular cells (Fig 1) Combined with the reduced lymph node weights this implies a great reduction in the absolute amount of lymphocytes *and* pyroninophilic cells in all lymph nodes examined In the medulla of the lymph nodes and in the *red*

TABLE

Splenic Weights (mg/100 g Body Weight) and Differential Cell Counts in Red Splenic Pulp Thyroxin-Induced Increase in the Amount of Pyroninophilic Cells Is Demonstrated

	No of animals	Splenic weights			Differential
		Whole spleen	Red pulp	White pulp	Lymphocytes
Sham operated controls	27	132 8 ± 4 4	108 1 ± 3 5	24 7 ± 1 4	21 31 ± 0 5
Sham-operated, thyroxin-treated	25	153 5** ± 5 8	126 4** ± 5 2	27 0 ± 1 5	19 33 ± 0 8
Thymectomized controls	34	130 2 ± 4 1	108 0 ± 3 4	22 2 ± 1 2	19 89 ± 0 89
Thymectomized thyroxin treated	24	142 9 ± 6 5	118 4 ± 5 5	24 5 ± 1 5	1 24 ± 0 31

* denotes significant effect of thyroxin * $p < 0.05$ ** $p < 0.01$, *** $p < 0.001$

splenic pulp, the decrease in lymphocyte content due to neonatal thymectomy was somewhat more pronounced in males than in females but this sex difference was not significant In contrast to the total amount of pyroninophilic cells in the medulla of the cervical lymph nodes, the relative plasma-cell content was slightly increased in the thymectomized animals, perhaps indicating that thymectomy has no reducing effect on the plasma cell content (*cf* *Azar et al* 1964)

Thyroxin-Treated Animals

Thymus Thyroxin treatment of the sham operated guinea-pigs increased the thymic weight by 5 per cent in the males and by 10 per cent in the females Thus, in the sham-operated animals, the sex difference in thymic weight was still more pronounced after thyroxin treatment ($p < 0.001$)

Spleen Thyroxin treatment produced a significant increase in splenic weight (red pulp only, not white Table 4) in the sham-operated guinea pigs ($p < 0.01$), but only an insignificant increase in the thymectomized animals In the red splenic pulp of both categories, thyroxin treatment caused a highly significant increase in the percentage of pyroninophilic cells, as well as in the number of these cells per unit of volume ($p < 0.001$) The total amount of pyroninophilic cells in the red splenic pulp in relation to body weight showed a similar increase in sham operated as well as thymectomized guinea-pigs ($p < 0.001$) The relative and absolute amounts of lymphocytes and reticular cells in the red splenic pulp were not significantly changed by thyroxin treatment (Table 4) The thyroxin induced increase in the amount of pyroninophilic cells in the red splenic pulp was greater in females than in males, this sex difference being more conspicuous in the thymectomized ani-

Sham Operated and Thymectomized Guinea Pigs Treated with Thyroxin or Saline A
Sham Operated as well as Thymectomized Animals Mean ± Standard Error

counts in red pulp (%)		No of cells per unit volume of red pulp			Comparable values of no of each cell type in whole red pulp related to body weight		
ronino lic cells	Reticular cells	Lymphocytes	Pyroninophilic cells	Reticular cells	Lymphocytes	Pyroninophilic cells	Reticular cells
5 08 + 0 31	73 11 ± 0 87	44 1 ± 2 0	10 2 + 0 6	147 8 ± 3 5	4791 ± 280	1123 ± 86	15985 ± 662
7 98*** + 0 16	72 49 ± 0 99	40 0 ± 1 9	16 3*** ± 0 9	118 7 ± 3 4	5044 ± 306	2088*** ± 171	18574* ± 910
4 60 + 0 33	75 50 ± 1 00	38 6 ± 2 5	8 7 ± 0 6	143 7 ± 3 0	4295 ± 333	970 ± 85	15702 ± 620
7 71** ± 0 56	75 04 ± 1 11	34 3 ± 1 8	15 2*** ± 1 1	149 0 ± 3 8	4163 ± 331	1858*** ± 187	17693 ± 946

mals than in the sham operated Thus, the thyroxin induced increase in the percentage of pyroninophilic cells, in the number of pyroninophilic cells per unit of volume and in the amount of pyroninophilic cells in whole red splenic pulp was greater in the female than in the male thymectomized animals ($p < 0.05$, $p < 0.05$ and $p < 0.001$, respectively) In the sham operated animals, the sex difference in the effect of thyroxin was significant only for the amount of pyroninophilic cells in whole red splenic pulp ($p < 0.05$)

The results prove that the influence of thyroxin on the cell composition of the red splenic pulp is almost identical in thymectomized and sham-operated animals, although the increase in weight of the spleen is less pronounced in the former

Lymph nodes After thyroxin treatment, all groups of lymph nodes both in sham-operated and thymectomized animals were increased in weight, although not significantly (Fig 2) In the *sham-operated animals* thyroxin treatment increased the lymphocyte content of the medulla and cortex of cervical and extremital nodes As regards plasma cells an appreciable percentage was found only in the medulla of cervical nodes where an increase was produced by thyroxin treatment In *the thymectomized animals*, the low ratios of lymphocytes/reticular cells and pyroninophilic cells/reticular cells were increased by thyroxin treatment in the medulla and cortex of the extremital nodes ($p < 0.05$ in the medulla) In the cervical lymph nodes, the ratio of lymphocytes/reticular cells was increased by thyroxin in the medulla but not in the cortex, whereas the ratio of pyroninophilic cells/reticular cells was slightly decreased in both medulla and cortex (none of the changes were significant) The percentage of plasma cells in the cervical medulla was not increased by thyroxin treatment

Thus, in the extremital lymph nodes, thyroxin treatment prevented

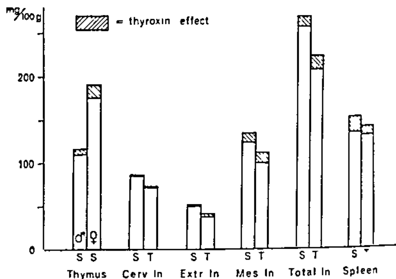


Fig 2

Effect of thyroxin on lymphatic tissue weights in guinea pigs thymectomized (T) or sham operated (S) at birth A similar stimulating effect of thyroxin on the normal tissue of the sham operated and on the involuted tissue of the neonatally thymectomized guinea pigs is demonstrated

most of the relative deficiency in lymphocytes and pyroninophilic cells seen in the thymectomized animals However, since the cervical and extremital nodes did not behave identically, no general conclusions are permissible regarding the influence of thyroxin on the relative cell composition of the lymph nodes of thymectomized animals

DISCUSSION

The observation of a greater thymic weight in female than in male guinea-pigs is in agreement with the report of *Andreasen* (1938), who also found a significant difference between the thymic weight in females and males, increasing with age In newborn and very young guinea pigs no such significant sex difference in thymic weight exists (*Gyllensten* 1953)

The present investigation has proved that the guinea pig does not differ qualitatively from the mouse, rat and golden hamster as regards the importance of an intact thymus for postnatal development of the lymphatic tissue Further, it is shown that the content of lymphocytes and pyroninophilic cells is more affected by the neonatal thymectomy than is the content of reticular cells As in the experiments of *Schooley & Kelly* (1964) in rats thymectomized at the age of 6–8 days, the splenic weight was not significantly altered by neonatal thymectomy

Inhibited growth of the lymphatic system is not seen in rats (*Schooley & Kelly* 1964) or guinea-pigs (*Ernstrom* 1965 a) thymectomized at the age of one month The inhibited growth in animals thymectomized at birth may be due to

1 Absence of thymocytes normally populating the lymphatic system
2 Absence of thymic humoral or cellular factors important to lymphoid differentiation and intrinsic growth of the lymphatic tissue
3 Absence of the thymus as an important centre for instruction of lymphocytes before proliferation in the lymphatic tissues
4 Increased production or decreased decay of lymphocytolytic steroids

Ad 1 and 2 Schooley & Kelly (1964) suggested that migrant thymocytes normally populating the lymphatic system postnatally are of greater importance than thymic factors stimulating postnatal lymphatic growth On the other hand, there is evidence of restoration and reconstitution of the lymphatic system in neonatally thymectomized mice by thymic tissue in Millipore chambers and by thymic homografts, without the occurrence of lymphoid chimerism (see reviews by *Miller* 1964, *Law et al* 1964)

Ad 1 and 3 Ford & Micklem (1963) found that the thymus of heavily irradiated mice was repopulated by injected bone marrow cells, but not by injected thymus or lymph node cells Furthermore, parabiosis experiments in mice have shown that the dividing cells in the normal thymus are slowly replaced by cells from the parabiotic partner (*Harris, Barnes & Ford* 1964), suggesting that the thymus receives stem cells from the bone marrow directing these cells into lymphoid differentiation (see *Metcalf* 1964)

Ad 4 Evidence against an effect of thymectomy on adrenocortical production of lymphocytolytic steroids has been provided by several authors (see *Fachet et al* 1962, *Tanaka* 1963) Steroid catabolism, however may be affected by thymectomy, since *Dougherty et al* (see review 1961) have reported that thymic tissue normally inactivates cortisol

The present finding of a similarly decreased amount of lymphocytes and pyroninophilic cells in the lymphatic organs of the thymectomized animals may be interpreted as a reduction in the number of pyroninophilic cells derived from small migrant thymocytes (*cf Fichtelius & Bryant* 1964) as well as decreased proliferation of pyroninophilic cells with decreased production of lymphocytes, due to the absence of thymic factors stimulating peripheral lymphopoiesis

The [illegible]

[illegible] humoral thymic factors or to lymphocyte populations as yet intact during the period directly following thymectomy Such a hypothesis is supported by the finding of a difference between the effect of exogenous thyroxin on lymphatic tissue weights shortly after and one month after thymectomy of young guinea pigs

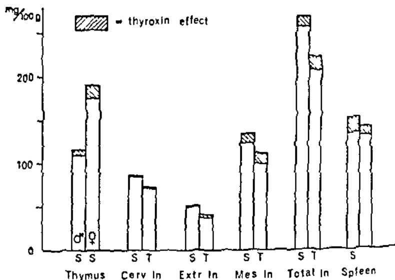


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thyroxin tended to prevent part of the lymphocyte deficiency caused by neonatal thymectomy, whereas no similar tendency was seen in the cell populations of the cervical nodes

The investigation has proved that neonatal thymectomy causes hypotrophy of the lymphatic system in guinea-pigs similar to that in *e g* mice and rats Furthermore, it is shown that thyroxin has similar effects on cell proliferation and cytomorphosis in neonatally thymectomized and sham operated guinea-pigs, at any rate in the spleen

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(*Ernstrom* 1965 a and b), but is complicated by the present finding that even after neonatal thymectomy, thyroxin treatment at the age of 41–50 days tends to prevent part of the lymphatic atrophy The latter effect of thyroxin agrees with the observation that steroid-involuted lymphatic tissue is stimulated to a certain degree by thyroxin, even in thymectomized animals (*Ernstrom & Gyllensten* 1965) The results should also be discussed with regard to the fact that involuted lymphatic tissue is more prompt to react to thyroxin stimulation than is normal lymphatic tissue (*Gregoire* 1942, *Barnholdt–von Euler et al* 1959, *Gyllensten* 1962), probably due to differences in the local homeostatic mechanisms

In accordance with previous results (*Ernstrom & Gyllensten* 1959), the present investigation reveals a highly increased number of pyroninophilic cells in the red splenic pulp 9 days after the first injection of thyroxin Furthermore, the results demonstrate that thyroxin produces almost identical changes in the differential cytology of the spleen in sham-operated and in thymectomized guinea-pigs, even if the increase in splenic weight is somewhat less pronounced in the thymectomized animals

The investigation has proved that neonatal thymectomy causes lymphatic hypotrophy in guinea pigs, when examined a sufficiently long time after operation, and that thyroxin-stimulated cell proliferation and cytomorphosis, at least in the red splenic pulp, are not necessarily dependent on an intact thymus or on any short lived humoral or cellular thymic factors

SUMMARY

Guinea-pigs were thymectomized or sham-operated at birth, and treated with thyroxin (50 µg/kg body weight injected every 3rd day) or saline from 41–50 days of age They were then killed Cervical, extremital and mesenteric lymph nodes were dissected quantitatively and weighed Histological examination with differential cell counts was performed on the different organs In the spleen, the relative amount of red and white pulp was determined by planimetry The following results were obtained

1 Neonatal thymectomy resulted in a significantly decreased amount of lymph-node tissue at the age of 50 days, but did not affect the growth and survival of the animals In all lymphatic organs examined, there was a much greater reduction in the amount of lymphocytes and pyroninophilic cells (although not of plasma cells) than of reticular cells

2 Treatment with thyroxin caused an increase in weight of the spleen and lymph nodes of sham-operated as well as thymectomized animals In the red splenic pulp, thyroxin induced a pronounced increase in the number of pyroninophilic cells irrespective of an intact thymus, although the increase in weight of the red pulp was significant only in the sham-operated guinea pigs In the extremital lymph nodes,

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THYROXIN STIMULATED VENOUS OUTPUT OF SMALL LYMPHOCYTES FROM THE THYMUS

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Received 17.xi.65

The thymic cortex has a much more intense proliferation of cells than the lymphatic tissue—higher mitotic counts (*Kindred* 1955 *Andreasen & Christensen* 1949, and others), greater incorporation of ^{32}P into DNA (*Andreasen & Ottesen* 1944, 1945, *Fichtelius* 1953, *Clark & Stoerk* 1956, *Lundin* 1958 and others), and higher incidence of labelled lymphocytes shortly after a single injection of ^{3}H thymidine (*Schooley, Bryant & Kelly* 1959, *Mims* 1962) Further evidence of an intense thymic production of lymphocytes remaining in the thymus for only a short time has been provided by *Everett, Rieke & Caffrey* (1964)

Thus there is no controversy about the intense production of lymphocytes in the thymus It is also evident that many thymocytes die within the organ (*Kindred* 1955) Whether thymocytes ever leave the thymus has been doubted (see *Gowans* 1964, *Metcalf* 1964 a, b) and the proportion between cells undergoing intrathymic death and possible, emigrating cells is not known An immigration of stem cells into the thymus is also probable since parabiosis experiments have shown a slow replacement of dividing thymic cells by cells derived from the para biotic partner (*Harris Barnes & Ford* 1964)

A previous investigation revealed a greater number of lymphocytes in efferent thymic blood than in carotid arterial or femoral venous blood, indicating a net emigration of lymphocytes from the thymus (*Ernstrom, Gyllensten & Larsson* 1965 *Ernstrom & Larsson* 1965 b) The present investigation is a further contribution to the solution of problems of migration of cells to and from the thymus

MATERIAL AND METHODS

A total of 83 young male guinea pigs weighing 200–280 g …
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[1] Some of the animals were identical with those in a previous investigation, performed at the same time as the present one (*Ernström & Larsson* 1965 b).

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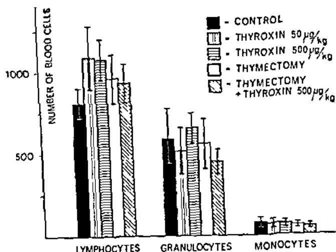


Fig 1
Number of lymphocytes granulocytes and monocytes in blood from the carotid artery in the different groups of guinea pigs Mean ± standard error

treated animals with intact thymus, the mean number of lymphocytes was markedly, although not significantly, increased, due to a great individual variation in the total white cell count (Fig 1)

The composition of the lymphocyte populations of thymic-vein blood and carotid artery blood was compared The mean MC of the lymphocytes in blood from the thymic vein was lower than that of the lymphocytes in blood from the carotid artery This difference was almost significant in normal controls, significant 6 hours after 50 µg of thyroxin/kg body weight and highly significant 6 hours after 500 µg/kg body weight in a single injection (Table 2) The mean MC of the lymphocytes in blood from the thymic vein and from the carotid artery was decreased in the thyroxin treated animals as compared with the corresponding values in the controls The decreases were significant only with the large dose of thyroxin and not with the small one (Table 2) The difference between thymic-vein blood and carotid artery blood and between blood of controls and thyroxin treated animals was most pronounced in the case of lymphocytes with 0 5 mitochondria per cell Table 3)

In view of the highly significant decrease in the mean MC and the significant increase in the percentage of lymphocytes with 0 5 mitochondria in blood from the carotid artery of the animals treated with the large dose of thyroxin, it could be tested whether the changes were due essentially to an increased output from the thymus of lymphocytes with low MC If so, this change in blood from the carotid artery observed 6 hours after the large dose of thyroxin would not occur in thymectomized animals

with NaCl. The other 12 were injected with the larger dose of thyroxin (500 µg/kg body weight) on the 12th day after operation.

All thyroxin treated animals in this experiment, thymectomized or with intact thymus, were examined 6 hours after the single injection of thyroxin. The investigative procedure was similar to that used in a previous experiment (*Ernström & Larsson* 1965 b), i.e. the animals were anaesthetized with Mebumal sodium, one large thymic vein and one of the carotid arteries were exposed and samples taken from each of the vessels almost simultaneously. Differential counts of blood smears and classification of lymphocytes by their mitochondrial content (MC) in supravitally stained preparations were performed on blood from the thymic vein and from the carotid artery (for technique see *Ernström & Larsson* 1961). The total number of white cells was determined in the carotid artery blood. Special interest was focused on the comparison between the differential mitochondrial counts of lymphocytes from the carotid artery and thymic vein blood in the same animals. These comparisons were made by coded experiments. The thoracic duct lymph was similarly examined in most of the animals (for technique see *Ernström & Larsson* 1965 b).

The results were analysed statistically by Student's t test. Statistical p values of < 0.05, < 0.01 and < 0.001 are denoted as almost significant, significant and highly significant, respectively. The statistical comparison of percentage and number of lymphocytes in blood from the thymic vein and the carotid artery was based on the differences found in the individual animals.

A mitochondrial content of < 11, 11–20 and > 20 mitochondria per cell is denoted as low, medium and high MC, respectively. As the mitochondrial content is correlated to the size of the lymphocytes, those with few mitochondria are small lymphocytes (*Wiseman* 1931, *Fichtelius & Larsson* 1961, *Ernström & Larsson* 1963).

RESULTS

The percentage of lymphocytes of total white cells in blood from the thymic vein exceeded that in blood from the carotid artery, both in normal controls and in animals 6 hours after a single dose of thyroxin (50 or 500 µg/kg). The thyroxin caused an increased percentage of lymphocytes in blood from the thymic vein as compared with findings in untreated controls (Table 1).

TABLE 1

Percentage of Lymphocytes of Total White Cells in Thymic Vein and Carotid Artery Blood in the Different Groups of Guinea Pigs. Mean ± Standard Error. TV = Thymic Vein, CA = Carotid Artery. A Thymic Arterio-Venous Difference in Percentage of Lymphocytes is Demonstrated

	No. of animals	Percentage of lymphocytes		
		TV	CA	TV–CA
Intact controls	25	69.0 ± 2.2	60.7 ± 2.5	8.4 ± 2.1 $p < 0.001$
Thyroxin 50 µg/kg	22	72.7 ± 2.5	64.9 ± 2.5	7.7 ± 2.8 $p < 0.05$
Thyroxin 500 µg/kg	12	72.3 ± 1.8	60.1 ± 2.8	12.2 ± 3.0 $p < 0.01$
Thymectomized controls	12		65.8 ± 4.0	
Thymectomized thyroxin 500 µg/kg	12		66.3 ± 3.1	

The absolute number of lymphocytes was determined in carotid artery blood. In the two experimental groups comprising thyroxin

Blood in Controls Thyroxin Treated (50 and 500 μg kg respectively) Thymectomized s after a Single Injection of Thyroxin) Mean ± Standard Error

ochondrial content Lymphocytes in %					Average no of mitochondria lymphocyte	p value of difference
11 15	16 20	21 30	> 30			
1 ± 1 4	7 8 ± 0 7	2 6 ± 0 5	0 1 ± 0 1		9 60 ± 0 20	$p < 0\,05$
1 ± 0 9	8 8 ± 0 8	3 3 ± 0 3	0 0 ± 0 0		10 10 ± 0 12	
4 + 1 8	6 9 ± 0 7	2 3 ± 0 3	0 1 ± 0 1		9 17 ± 0 15	$p < 0\,01$
1 + 1 4	8 5 ± 0 9	3 0 ± 0 5	0 1 ± 0 1		9 95 ± 0 21	
2 ± 1 1	5 5 ± 0 7	1 8 ± 0 4	0 7 ± 0 3		8 69 ± 0 15	$p < 0\,001$
9 + 1 1	7 0 + 0 7	2 9 ± 0 2	0 1 ± 0 1		9 49 ± 0 10	
1 1 ± 1 2	8 3 ± 0 7	3 3 ± 0 3	0 3 ± 0 2		10 20 ± 0 11	$p < 0\,001$
0 + 1 0	11 2 ± 0 8	4 4 + 0 3	0 3 ± 0 2		10 95 ± 0 09	

oxin/kg body weight and examined 6 hours later, did not show a decrease in mean MC of the lymphocytes in blood from the carotid artery (and no increase in percentage of lymphocytes with 0–5 mitochondria) as found in the identically treated animals with intact thymus (Tables 2 and 3) On the contrary the mean MC was increased ($p < 0\,001$) and the percentage of lymphocytes with 0–5 mitochondria decreased ($p < 0\,001$)

The absolute number of lymphocytes of different classes was determined in blood from the carotid artery (Fig 2) As for the percentage of lymphocytes the most conspicuous changes were seen in the lymphocytes with 0 5 mitochondria per cell (Fig 2) Thus in this class, the large dose of thyroxin caused a significant increase in the number of lymphocytes *in the intact animals* but an almost significant decrease in the thymectomized ones The difference in effect of thyroxin on the number of lymphocytes with 0 5 mitochondria per cell in blood from the carotid artery of intact and thymectomized animals was highly significant (Table 3)

The greater number of lymphocytes in the efferent than in the afferent thymic blood makes it possible to assume that no or few lymphocytes with high MC are selectively eliminated from the blood during its passage through the thymus On this assumption the minimum number of thymic lymphocytes added per mm³ of afferent thymic blood can be calculated from the distribution of lymphocytes with different MC in afferent and efferent thymic blood Such hypothetical calculation shows an output of thymic lymphocytes with low MC in normal controls and a still greater output 6 hours after a single injection of 50 or 500 μg of thyroxin/kg body weight (Table 4)

The lymphocytes in the thoracic-duct lymph were classified by their

TABLE

Comparison Between Differential Counts of Lymphocytes in Carotid Artery and Thymic ... and Thymectomized + Thyroxin Trea'ed (500 µg/kg) Guinea Pigs (6

		No of animals	Classification of lymphocytes	
			0 5	6 10
Intact controls	Thymic-vein blood	12	16 9 ± 1 6	48 5 ± 1 9
	Carotid artery blood	12	13 6 ± 1 0	47 3 ± 1 1
Thyroxin treated, 50 µg/kg	Thymic vein blood	12	21 5 ± 0 9	45 8 ± 1 9
	Carotid artery blood	12	14 8 ± 1 3	47 5 ± 1 5
Thyroxin treated 500 µg/kg	Thymic vein blood	12	24 9 ± 1 0	49 0 ± 1 3
	Carotid artery blood	12	18 5 ± 0 9	47 6 ± 1 0
Thymectomized controls	Carotid artery blood	12	13 5 ± 0 5	46 5 ± 0 8
Thymectomized, thyroxin-treated, 500 µg/kg	Carotid artery blood	12	9 6 ± 0 6	44 6 ± 1 2

The lymphocyte population in blood from the carotid artery of thymectomized controls (12 days after operation) did not differ from that of intact animals (in agreement with previous results *Ernstrom & Larsson* 1965 a) The thymectomized animals, treated with 500 µg of thyr-

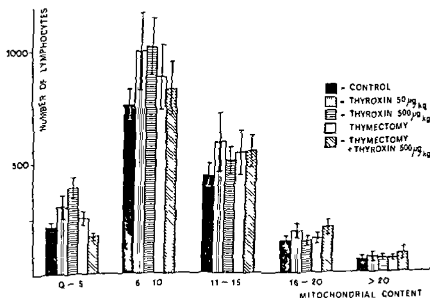


Fig 2

Number of lymphocytes with different mitochondrial content in blood from the carotid artery of controls thyroxin treated (50 and 500 µg/kg b w respectively), ... + thyroxin treated (500 µg kg b w) guinea pigs thyroxin) Mean ± standard error An ncreased ... ed by low mitochondrial content is demonstrated animals but a decrease after thyroxin treatment mectomized animals

TABLE 4

Hypothetical Calculation of the Relative Number of Thymic Lymphocytes of Different Classes (Classified by Mitochondrial Content) Added to the Afferent Arterial Blood per mm3 in Normal and Thyroxin Treated Guinea Pigs (6 Hours after a Single Injection of Thyroxin) TV = Thymic Vein CA = Carotid Artery*

	Proportion between no. of lymphocytes with different mitochondrial content								No. of lymphocytes mm³	No. of lymphocytes mm³ added			
	TV				CA				CA (=TV)	TV – CA			
MC	0 5	6 10	11 15	> 15	0 5	6 10	11 15	> 15	> 15	0 5	6 10	11 15	0 15
Controls	1 6	4 6	2 3	1	1 1	3 9	2 2	1	201	101	141	20	262
Thyroxin treated, 50 µg kg	2 3	4 9	2 5	1	1 3	4 1	2 3	1	257	257	206	51	514
Thyroxin treated 500 µg kg	3 1	6 1	2 3	1	1 9	4 8	2 4	1	213	256	277	—21	512

* The calculation is based on the assumption that no significant number of lymphocytes with MC > 15 in the afferent arterial blood is retained in the thymus. The proportions between the number of lymphocytes with different MC are obtained from Table 2, using the number of lymphocytes with MC > 15 as unit.

TABLE 3

Percentage of Lymphocytes with 0.5 Mitochondria (Small Lymphocytes) of Total Lymphocytes in Thymic Vein and Carotid Artery Blood and Number of Lymphocytes with 0.5 Mitochondria per mm³ of Carotid Artery Blood. Mean ± Standard Error. TV = Thymic Vein, CA = Carotid Artery. A Thyroxin Induced Increase in the Thymic Arterio-Venous Difference in Percentage of Small Lymphocytes is Shown. A Thyroxin Induced Increase in the Number of Small Lymphocytes in Carotid Artery Blood is Demonstrated in Intact Guinea Pigs but a Decrease in Thymectomized Guinea Pigs

	No. of animals	Percentage of small lymphocytes			No. of small lymphocytes/mm³
		TV	CA	TV–CA	CA
Intact controls	12	16.92 ± 1.58	13.58 ± 0.96	3.34 ± 1.85	214 ± 22
Thyroxin treated 50 µg/kg	12	21.50 ± 0.89*	14.83 ± 1.30	6.67 ± 1.57 p < 0.001	305 ± 50
Thyroxin treated 500 µg/kg	12	24.92 ± 0.97***	18.50 ± 0.91**	6.42 ± 1.33 p < 0.001	390 ± 45**
Thymectomized controls	12		13.50 ± 0.53		248 ± 31
Thymectomized thyroxin treated 500 µg/kg	12		9.78 ± 0.62***		169 ± 17*
Difference in effect of thyroxin on intact and thymectomized animals			8.84 ± 1.56 p < 0.001		255 ± 62 p < 0.001

* p < 0.05 ** p < 0.01 *** p < 001 denotes significant effect of thyroxin on intact and thymectomized animals respectively

mitochondrial content (Table 5) The small dose of thyroxin caused a significant increase in the percentage of lymphocytes with 11–15 mitochondria per cell, and a corresponding decrease in those with lower and higher MC Thymectomy caused similar changes in the relative composition of the lymphocyte population of the thoracic-duct lymph The large dose of thyroxin produced no significant changes in the lymph, neither in intact nor in thymectomized animals As no measurements of the lymph flow were made, the relative changes do not permit direct conclusions Thyroxin induces an increased number of lymphocytes with medium MC in the blood (*Ernstrom & Larsson* 1965 a), while thymectomy reduces the output of lymphocytes through the thoracic duct (*Reinhardt & Yoffey* 1956) Thus it seems possible that the thyroxin induced changes in the lymphocyte population are due to an increased output of lymphocytes with medium MC, and that the changes induced by thymectomy are due to a decreased output of lymphocytes with low and high MC

DISCUSSION

The comparison between the afferent thymic blood taken from a carotid artery and the efferent thymic blood taken from a thymic vein reveals a greater mean MC in the lymphocytes of the afferent blood This difference may depend either on a selective retention of lymphocytes with high MC in the thymus, or on an output from it of lymphocytes with low MC The percentage of lymphocytes in blood from thymic veins exceeds that in blood from a carotid artery (Table 1) It was demonstrated in a previous investigation that this is due to an addition of lymphocytes to the blood, and not to an elimination of other [illegible]

[illegible] do not settle in the adult thymus to any appreciable extent (*Gowans* 1964) Nevertheless, it cannot be stated that *no* lymphocytes enter the thymus, as *Harris et al* (1964) have provided evidence of a slow replacement of thymic cells by stem cells from the circulation From a quantitative point of view, however, the venous output of lymphocytes from the thymus exceeds such immigration of cells into it (*Ernstrom & Larsson* 1965 b)

An increased percentage of lymphocytes with low MC ("small" lymphocytes) occurs in blood from a thymic vein 6 hours after a single injection of thyroxin (50 µg/kg body weight) This finding (*Ernstrom & Larsson* 1965 b), suggesting a thyroxin stimulated output of "small" thymic lymphocytes, motivated the present comparison between afferent and efferent thymic blood in thyroxin-treated animals The suggestion was, in fact, confirmed by the present finding that the difference between the percentage of lymphocytes with low MC (especially the "smallest" lymphocytes characterized by 0–5 mitochondria) in blood from the thymic vein and from the carotid artery was greater in thyr-

TABLE 5

Differential Counts of Lymphocytes in the Thoracic Duct Lymph in Controls Thyroxin-Treated (50 and 500 µg/kg respectively), Thymectomized and Thymectomized + Thyroxin Treated (500 µg/kg) Guinea Pigs (6 Hours after a Single Injection of Thyroxin) Mean ± Standard Error

	No of animals	Classification of lymphocytes by mitochondrial content Lymphocytes in %						Average no of mitochondria/ lymphocyte
		0–5	6–10	11–15	16–20	21–30	> 30	
Intact controls	21	7.5 ± 0.4	29.0 ± 1.2	38.8 ± 1.3	15.5 ± 1.0	7.8 ± 0.6	1.5 ± 0.3	12.85 ± 0.19
Thyroxin treated 50 µg/kg	14	5.2 ± 0.4***	29.0 ± 1.3	44.3 ± 1.2**	15.1 ± 1.0	5.5 ± 0.3**	0.9 ± 0.3	12.63 ± 0.12
Thyroxin treated 500 µg/kg	10	8.3 ± 1.2	31.1 ± 1.2	37.9 ± 1.1	15.2 ± 1.3	6.8 ± 0.7	0.7 ± 0.5	12.35 ± 0.31
Thymectomized controls	10	5.7 ± 0.5	27.0 ± 1.6	45.7 ± 1.7++	14.6 ± 1.1	6.5 ± 0.6	0.6 ± 0.2+	12.74 ± 0.21
Thymectomized thyroxin treated 500 µg/kg	12	4.8 ± 0.5	25.5 ± 1.5	45.8 ± 1.2	15.8 ± 1.0	7.3 ± 0.4	0.8 ± 0.2	13.02 ± 0.20

* denotes significant effect of thyroxin ** $p < 0.01$ *** $p < 0.001$
+ denotes significant effect of thymectomy + $p < 0.05$, ++ $p < 0.01$

mitochondrial content (Table 5) The small dose of thyroxin caused a significant increase in the percentage of lymphocytes with 11–15 mitochondria per cell, and a corresponding decrease in those with lower and higher MC Thymectomy caused similar changes in the relative composition of the lymphocyte population of the thoracic-duct lymph The large dose of thyroxin produced no significant changes in the lymph, neither in intact nor in thymectomized animals As no measurements of the lymph flow were made, the relative changes do not permit direct conclusions Thyroxin induces an increased number of lymphocytes with medium MC in the blood (*Ernstrom & Larsson* 1965 a), while thymectomy reduces the output of lymphocytes through the thoracic duct (*Reinhardt & Yoffey* 1956) Thus it seems possible that the thyroxin induced changes in the lymphocyte population are due to an increased output of lymphocytes with medium MC, and that the changes induced by thymectomy are due to a decreased output of lymphocytes with low and high MC

DISCUSSION

The comparison between the afferent thymic blood taken from a carotid artery and the efferent thymic blood taken from a thymic vein reveals a greater mean MC in the lymphocytes of the afferent blood This difference may depend either on a selective retention of lymphocytes with high MC in the thymus, or on an output from it of lymphocytes with low MC The percentage of lymphocytes in blood from thymic veins exceeds that in blood from a carotid artery (Table 1) It was demonstrated in a previous investigation that this is due to an addition of lymphocytes to the blood, and not to an elimination of other white cells (granulocytes and monocytes) from the blood during its passage through the thymus (*Ernstrom & Larsson* 1965 b) Furthermore, labelled lymphocytes do not settle in the adult thymus to any appreciable extent (*Gowans* 1964) Nevertheless, it cannot be stated that *no* lymphocytes enter the thymus, as *Harris et al* (1964) have provided evidence of a slow replacement of thymic cells by stem cells from the circulation From a quantitative point of view, however, the venous output of lymphocytes from the thymus exceeds such immigration of cells into it (*Ernstrom & Larsson* 1965 b)

An increased percentage of lymphocytes with low MC (" — " [illegible]

[illegible] suggesting a thyroxin-stimulated output of "small" thymic lymphocytes, motivated the present comparison between afferent and efferent thymic blood in thyroxin-treated animals The suggestion was in fact confirmed by the present finding that the difference between the percentage of lymphocytes with low MC (especially the "smallest" lymphocytes characterized by 0 5 mitochondria) in blood from the thymic vein and from the carotid artery was greater in thyr-

oxin-treated animals than in normal animals (Table 3) Moreover, thyroxin (especially the large dose) increased the percentage of these "small" lymphocytes in blood from the thymic vein (Table 3) The results strongly favour the view that injection of thyroxin increases the output of "small" lymphocytes (with low MC) from the thymus within 24 hours (*Ernstrom* 1963) The effect of the large dose of thyroxin also suggests that this output of small lymphocytes increases with the dose of thyroxin administered Thus, the thymic atrophy, known to occur in animals treated with large doses of thyroxin (*Schultze* 1933, *Andreasen* 1937, 1938), is due partly to an increased delivery of "small' lymphocytes to the blood An increased intrathymic death of thymocytes may also contribute to this atrophy

After 6 hours, the large dose of thyroxin produced an increased percentage and an increased number of "small" lymphocytes in blood from the carotid artery of intact animals In thymectomized animals, this change did not occur after the same dose of thyroxin (Fig 2 and Table 3) This is further strong evidence of the mobilization of "small" lymphocytes from the thymus within 6 hours of administration of thyroxin The results also suggest that the "small" lymphocytes appearing in blood from the carotid artery within 6 hours of a thyroxin injection derive essentially from the thymus Contrary to the finding in animals with an intact thymus, the percentage of blood lymphocytes with low MC was decreased in the thymectomized animals 6 hours after thyroxin One plausible explanation is a rapid consumption of "small circulating lymphocytes with low MC (lysis or transformation into larger lymphocytes with higher MC) due to the thyroxin treatment with a compensatory increased output of "small" lymphocytes from the thymus in intact but not in thymectomized animals (*cf Ernstrom & Larsson* 1965 a) The comparison between the absolute number of blood lymphocytes with different MC in the experimental groups also discloses that, 6 hours after the injection of thyroxin, the number of "small blood lymphocytes is increased in the animals with an intact thymus but decreased in the thymectomized animals This favours the theory of an increased appearance in the blood of "small" lymphocytes derived from the thymus and, simultaneously, an increased disappearance from the blood of such cells in the thyroxin-treated animals

SUMMARY

The lymphocyte populations in blood from a thymic vein and a carotid artery, and in thoracic duct lymph were compared in normal thyroxin-treated and thymectomized male guinea-pigs The lymphocytes were classified according to their mitochondrial content (MC) The investigation was performed 6 hours after the administration of 50 or 500 μg of thyroxin/kg body weight and 12 days after thymectomy The results and conclusions were as follows

1 The percentage of lymphocytes of total white cells is greater in blood from the thymic vein than in blood from the carotid artery. Especially the lymphocytes with low mitochondrial content ("small" lymphocytes) are more numerous in thymic vein blood than in afferent thymic blood.

2 Thymectomy alone causes no significant change in the number of blood lymphocytes, nor in the composition of the blood-lymphocyte population 12 days after operation.

3 Treatment with thyroxin produces a slight increase in the percentage of lymphocytes in blood from the thymic vein after 6 hours. Of the total number of lymphocytes, the percentage of "small" ones increases—more after the large dose of thyroxin than after the small one. The arterio-venous difference in relative number of "small" lymphocytes is greater in the thyroxin-treated than in the normal animals.

After the large dose of thyroxin, an increased number of "small" lymphocytes is demonstrated in blood from the carotid artery of intact animals, but not in identically treated thymectomized animals in which, on the contrary, thyroxin produces a decrease in the number of "small" lymphocytes.

Evidence is thus given of a thyroxin stimulated output of "small" lymphocytes from the thymus to the blood, and indications of a thyroxin increased disappearance of "small" lymphocytes from the blood outside the thymus.

4 Thyroxin in low dose or thymectomy causes an increased percentage of lymphocytes with medium mitochondrial content in the thoracic duct lymph. The thyroxin induced increase probably reflects an actual increased output of such lymphocytes, whereas the change induced by thymectomy may as well be due to a decreased output of lymphocytes with low and high mitochondrial content.

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MITOTIC STUDIES IN THYROXIN-STIMULATED THYMO-LYMPHATIC TISSUE

An Investigation in Guinea-Pigs with the Colchicine Technique

By

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Previous investigations have revealed increased growth of the thymo-lymphatic organs, with an increased incidence of large basophilic cells (known to be very frequent in rapidly proliferating lymphoid tissues), in guinea pigs treated with thyroxin (*Gyllensten* 1953, *Ernström & Gyllensten* 1959, *Ernström* 1963, 1965). In the present paper an account is given of a further study on the proliferation of lymphoid cells in thyroxin treated guinea pigs, using colchicine as a stathmokinetic drug.

Several requirements must be fulfilled for a proper interpretation of colchicine experiments (see *Eigsti & Dustin* 1955, *Leblond* 1959). Although objections to the method may thus be raised, it has been widely used in cytokinetic studies, and most authors have concluded that counts of colchicine blocked mitosis give a minimum figure for mitotic rate over the period of action of the drug (see e g *Leblond & Walker* 1956, *Dustin* 1959). Special difficulties as regards interpretation arise when colchicine is used for research on hormone stimulated tissues. The number of cells entering mitosis must not increase considerably during the period of action of colchicine—if so, a too low mitotic duration is erroneously calculated. Despite this limitation the colchicine method has provided much information about hormone-stimulated growth, e g of the accessory sex organs due to gonadotropic and gonadal hormones, of the adrenal cortex due to adrenocorticotropic hormone and of the thyroid gland due to thyrotropic hormone (see *Eigsti & Dustin* 1955).

The present study is a comparison between the mitotic rates and durations in the thymo-lymphatic organs of normal and thyroxin-treated guinea pigs.

MATERIAL AND METHODS

In a preliminary experiment different doses of colchicine were tested. A dose of 1 mg/kg body weight was most favourable. Higher doses resulted in a decreased number of cells in the metaphasic stage—increased pyknosis was recorded instead

Most of the dividing cells in the thymus of guinea pigs injected with colchicine were arrested in metaphase—a small fraction of cells were in ana or telophase The latter cells had escaped the colchicine blockade or had passed the metaphasic stage before the colchicine blockade had been established Less probable is the presence of cells with an extremely long duration of the ana and telophases exceeding 4 hours (*cf* *cold acclimated animals—Héroux 1960*)

Diurnal variations in mitotic activity have been demonstrated in various organs (*Bullough* 1948 *Bertalanffy* 1951 *Halberg et al* 1958) In the present experiment all animals were sacrificed between 12 a m and 1 p m

Young male guinea pigs weighing 200 270 g were used They were divided into four groups (number of animals in brackets)

Controls (9)

Colchicine treated controls (12)

Thyroxin treated (11)

Thyroxin treated colchicine-treated (11)

A single dose of thyroxin was given (100 µg/kg b w s c in the dorsum Thyroxin® Hoffman La Roche) between 4 and 5 p m Colchicine (N desacetyl N methyl colchicine 1 mg/kg b w i p Colcemid® Ciba) was injected 16 hours later, *i e* between 8 and 9 a m on the next morning The animals were sacrificed exactly 4 hours after the injection of colchicine (*i e* 20 hours after the thyroxin injection) The thyroxin treated animals not given colchicine were also sacrificed 20 hours after the thyroxin injection *i e* corresponding to the end point of colchicine action in the colchicine treated animals

The animals were killed by a blow on the neck and dissected immediately The thymic lobes, the paratracheal lymph nodes and the spleen were fixed in Carnoy s solution embedded in paraffin cut into 7µ thick sections and stained with methyl green and pyronine at pH 4 8

The thymic cortex the red splenic pulp the white splenic pulp the diffuse lymph node cortex and the germinal centres in the lymph node cortex of the animals in the four groups were examined microscopically (760 ×) The examination was made with coded specimens In each specimen the number of mitoses was counted in 100 fields of view, defined by a circle in the ocular—each such field comprising 150–800 cells (the cell density was lowest in the red splenic pulp and highest in the thymic cortex) The fields were taken at random The total number of cells (reticular
f different size) was counted in 2 such
ade between dividing cells of different
rage mitotic index was calculated for
d
rrests all metaphases the number of
e number of cells entering mitosis
between injection and sacrifice and the following formulas can be deduced (see *e g* *Leblond* 1959)

$$m = \frac{pt}{q}, \qquad r = \frac{100m}{p},$$

in which m = mitotic duration t = time between injection of colchicine and sacrifice, p = normal percentage of mitosis (without colchicine) q = percentage of colchicine blocked metaphases and r = turnover time (time for 100 per cent renewal) The formulas were used for calculation of comparable (but not real) mitotic rates and mitotic durations in the normal and thyroxin treated guinea pigs

RESULTS

The number of mitotic cells per 1000 cells (mitotic index) was highest in the germinal centres of the lymph-node cortex intermediate in the thymic cortex and white splenic pulp and lowest in the diffuse lymph-node cortex and red splenic pulp The mitotic cells in the thymus were most frequent in the peripheral cortex

Although thyroxin treatment did not produce any significant changes in the mitotic indices, its administration (without colchicine) tended

TABLE 1

Mitotic Indices (Mitotic Cells/1000 Cells) in the Thymo-Lymphatic Organs of Thyroxin Treated Guinea Pigs and Controls with and without Accumulation of Mitotic Cells with Colchicine. Calculations of Turnover Time and Mitotic Duration Demonstrate a Decreased Turnover Time in Thymus and Lymph Nodes and a Decreased Mitotic Duration in Thymus, Spleen and Lymph Nodes in the Thyroxin Treated Animals. Mean ± Standard Error

	No. of animals	Thymus	Spleen		Lymph nodes	
		Cortex	Red pulp	White pulp	Diffuse cortex	Germinal centres
Controls	9	4.82 ± 0.38	1.23 ± 0.13	3.39 ± 0.41	0.84 ± 0.02	11.88 ± 1.09
Colchicine treated controls	12	9.49 ± 2.91	2.26 ± 0.43	5.03 ± 0.98	1.92 ± 0.42	23.15 ± 4.61
Thyroxin treated	11	4.19 ± 0.27	0.97 ± 0.10	2.82 ± 0.37	0.65 ± 0.06	11.43 ± 0.05
Thyroxin and colchicine treated	11	14.45 ± 3.14	2.18 ± 0.31	5.18 ± 0.48	2.34 ± 0.52	35.50 ± 7.16
Turnover time in controls (hours)		422	1770	710	2083	173
Turnover time in thyroxin treated (hours)		277	1835	772	1709	113
Mitotic duration in controls (hours)		2.03	2.18	2.41	1.75	2.05
Mitotic duration in thyroxin treated (hours)		1.16	1.78	2.18	1.11	1.33

TABLE 2

Cell Density (Cells/Field of View) in the Thymo-Lymphatic Organs of Thyroxin Treated Guinea Pigs and Controls with and without Accumulation of Mitotic Cells with Colchicine. Mean ± Standard Error

	No. of animals	Thymus	Spleen		Lymph nodes	
		Cortex	Red pulp	White pulp	Diffuse cortex	Germinal centres
Controls	9	707 ± 20	171 ± 9	285 ± 7	322 ± 8	252 ± 13
Colchicine treated controls	12	710 ± 20	184 ± 5	291 ± 5	333 ± 9	247 ± 7
Thyroxin treated	11	729 ± 13	181 ± 6	304 ± 4*	356 ± 10*	242 ± 9
Thyroxin and colchicine treated	11	741 ± 14	186 ± 6	301 ± 6	341 ± 10	231 ± 7

* $p < 0.05$. Almost significant effect of thyroxin

to decrease them in all organs studied (Table 1). When, on the contrary, the mitotic cells were accumulated by colchicine, the thyroxin treated animals showed an increased mitotic index in the thymic cortex (+ 52 per cent), as well as in the lymph-node cortex (both germinal centres and diffuse cortex, + 53 and + 22 per cent, respectively)

The cell density was greatest in the thymic cortex and smallest in the red splenic pulp Thyroxin slightly increased the cell density in the thymic cortex, the spleen and the diffuse lymph-node cortex, but not in the germinal centres (Table 2)

The finding of an unchanged or possibly decreased mitotic index in the thyroxin-treated animals (not given colchicine), despite an apparently increased number of cells entering mitosis in the thymic cortex and germinal centres (as judged by the colchicine-treated animals), can be explained by thyroxin having a shortening effect on mitotic duration The calculations of mitotic duration and average turnover time in the cell populations studied were based on a time of action of colchicine of 4 hours Although this is an overestimation (see *Leblond* 1959, *Dustin* 1959)—the real time of action is probably about 3½ hours—it is of no importance for the comparison between the experimental groups As seen in Table 1, the calculated mitotic duration, in all five cell populations studied, were decreased in the animals given a single dose of thyroxin

DISCUSSION

A prerequisite for the present investigation is that colchicine itself does not interfere within 4 hours with the mitotic rate of the thymo lymphatic tissues studied No evidence of such an influence exists, even if a late effect of colchicine (after more than 8 hours) is lymphocytosis (*Dixon & Malden* 1908, *Lits* 1933, 1936, *Fagraeus & Gormsen* 1953 and others), apparently due to a mobilization of cells (at this time, the thymo-lymphatic organs are reduced in weight, and the incorporation of ^{3}P into RNA and DNA is diminished, see *Clark & Stoerk* 1956)

Furthermore, the arresting effect of colchicine on mitosis must not be modified by the thyroxin treatment, e g by a change in the catabolism or excretion of the drug As thyroxin is known to stimulate the decay of several metabolites, a thyroxin stimulated excretion of colchicine seems possible Such influence, however, would decrease the accumulation of mitotic cells in the thyroxin-treated animals, and thus cannot invalidate the conclusion that the mitotic rate is higher or the mitotic duration has shortened in these animals

The increased accumulation of mitotic cells and the increased cell density in the thymus during the period covering 16 20 hours after a single injection of thyroxin is in conformity with the cytological evidence of a stimulated proliferation of thymic cells within 24 hours of administration of the hormone (*Ernstrom* 1963)

The calculated average mitotic duration in all the cell populations examined was shorter in the thyroxin treated animals than in the controls. This implies that thyroxin may decrease the duration of mitosis in thymo-lymphatic cells (and perhaps in all dividing cells in the body). Such shortened mitotic duration explains the discrepancy between increased growth (increase in weight) and essentially unchanged or possibly decreased mitotic index observed in the thymo-lymphatic organs of thyroxin treated animals. It must be borne in mind, however, that great caution must be observed as regards the interpretation of a colchicine experiment.

SUMMARY

The mitotic indices in five parts of the thymo-lymphatic tissues (thymic cortex, red and white splenic pulp, diffuse lymph node cortex, germinal centres in lymph node cortex) were determined in normal and thyroxin treated guinea pigs with and without accumulation of colchicine-arrested mitotic cells. The determinations were made 20 hours after a single injection of thyroxin (100 μg/kg) and 4 hours after a single injection of colchicine (1 mg/kg).

Comparable values of average turnover time and mitotic duration in normal and thyroxin treated animals were calculated from the results. In the thyroxin treated animals the turnover time was decreased in the thymic cortex and germinal centres, whereas the mitotic duration was decreased in all five cell populations examined.

The experiment suggests that thyroxin has a shortening effect on mitotic duration in the thymo-lymphatic organs. It is emphasized, however, that due caution should be exerted in the interpretation of a colchicine experiment.

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MARGINAL GLIONEURAL HETEROTOPIAS OF THE CENTRAL NERVOUS SYSTEM

By

ARNE BRUN

Received 8 x 65

Marginal glioneural heterotopias of the central nervous system [*syn e g* heterotopic glial nests in the subarachnoidal space (7), ectopic glial nodules (8 9), nests of neuroglial tissue in the meninges (24)] are widely believed to be malformations, but opinions still differ on their origin Multipotent meningeal cells (15) and subarachnoidally implanted embryonal cells (11) have been considered possible sources Some authors (6 23) incline to the view that the glial nests arise from a persistent *superfizielles Kornerschicht* of Ranke (18) According to another similar theory, the glial nests are formed by protrusion of a luxuriant marginal glial tissue (7)

The following report of a case with such glial nests showed histological features, which may provide a clue to their origin The development of these features has been the subject of a previous study (3, 4)

REPORT OF CASE

The patient one of two female twins weighed 4,500 grams at birth No consanguinity or hereditary neurological diseases were known The other twin developed normally The patient did poorly and on admission to hospital at the age of 5 months (1 month before her death) she weighed 4 000 grams The circumference of the head was 34 cm The muscular tone of the arms and legs was increased A corneal opacity bilateral *coloboma* of the choroid and occipital craniotabes were diagnosed Hypothermia developed the circulation failed and the patient died at 6 months of age

tut pay

... the medulla

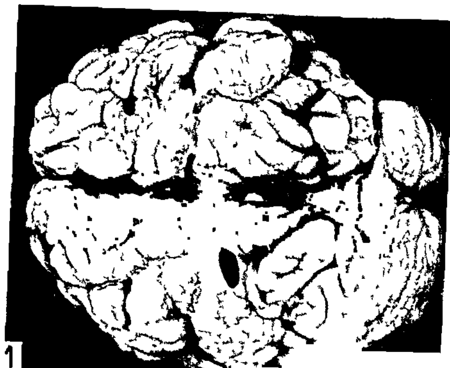
inferior olives were prominent owin-

parietal lobes showed two symmetric

The left opening was 0.5 1 cm in diameter and provided an open communication

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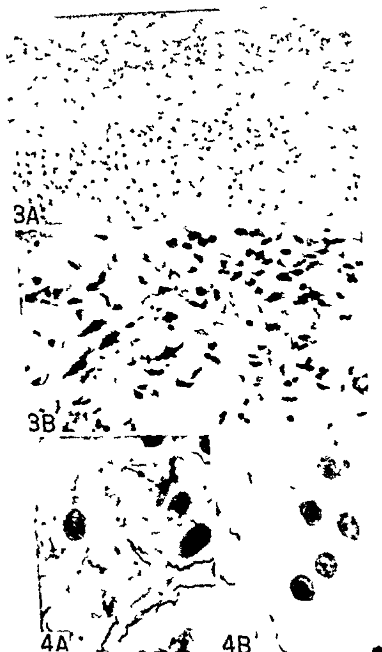




Figs 1 2

Fig 1 Brain seen from above Note symmetric pori and micropolygyric areas Shortened parieto occipital lobes exposing the cerebellum

Fig 2 Top of left hemisphere with porus seen from below Note micropolygyria heterotopias in the white matter and greyish tissue in the Sylvian fissure at arrow



Figs. 3-4

Fig. 3. A. Molecular layer showing subpial cellular layer while deep portions are relatively poor in cells. Nissl × 100. B. Higher magnification of subpial cellular layer with nerve cells among glial cells. Nissl × 400.

Fig. 4. A and B. Subpial cells with neurite and multiple nucleoli. Bodian × 1000.

with the ventricular system, while the right one was slightly smaller and partly closed by a fine membrane The tissue around the pori was smooth and tough The surface configuration of the base of the brain of the frontal and occipital lobes as well as of the medial surface of the hemispheres was normal

The insulæ were *incompletely covered owing to hypoplasia of the operculae* of the temporal frontal and centro parietal lobes The cortex here and in the insulae contained small crowded gyri but was of normal consistence These changed cortical areas were largely covered bilaterally with a superficial greyish tissue extending from the centro parietal operculum down to the olfactory trigone On the cut surface the tissue layer was 5 mm at its widest part tapering off towards its periphery (Fig 2) The cortical plate appeared unusually wide and it had a micro polygyric pattern Underneath it the centrum semiovale was reduced in width and contained multiple rounded heterotopic islands of grey matter which sometimes *bulged into the ventricular system* This was abnormally wide the callosal body was unusually thin The basal ganglia were well shaped The anterior limb of the internal capsule was abnormally thin on both sides, and the posterior limb was barely discernible The cut surface of the cerebellum appeared normal

Specimens were removed from various parts of the brain and studied some of them in serial sections of large tissue blocks from the fronto temporal lobes with the insula or in sections from a whole hemisphere

The sections were stained with hematoxylin eosin van Gieson Nissl Mahon Bodian PTAH, Cajal Silver, Cajal Gold Sudan Red and reticulin fibril staining *according to Soul*

Microscopic findings Only slight structural abnormalities were found in specimens from macroscopically normal areas of the cortical plate

In the insulæ and surrounding opercular areas however, the cortical plate was narrow but thrown into folds forming small crowded superimposed gyri giving the gross impression of a widened cortical plate The cortex showed sometimes 4 and sometimes 6 laminae but often complete disorganization The molecular layer was however, always identified and followed the complicated gyral pattern Sometimes the superficial cortical layers formed small nodular elevations projecting into the *molecular layer Within malformed areas the deeper parts of* the molecular layer contained few cells whereas the subpial strata showed clusters or a layer of cells separated from the pia by a narrow cell free zone (Fig 3 a)

The picture was dominated by glial cells but also exhibited triangular nerve cells (Fig 3 b) and more fusiform or elongated Cajal cells In addition many primitive cells were seen they were well outlined and had small round or pear shaped dark nuclei often containing two or more nucleoli (Figs 4 b 12 b) The cytoplasm was scanty and a process resembling a neurite sometimes extended from one of the cell poles (Fig 4 a) The tangential plexus was sometimes abnormally dense and well myelinated

The superficial cellular portions of the molecular layer were often continuous with the above described tumor like tissue [illegible] areas however they were lined by a pial membrane interr[illegible] of tissue often containing a vessel especiall[illegible] like excrescences a *subpial cellular layer* i[illegible] into the periphery of the tumour like tissue (Figs 6 12 a) The tumour like superficial tissue was rather rich in immature cells mainly spongioblastic and neuroblastic (Fig 7) The intercellular background substance had the same appearance as that of the molecular layer An increased number of vessels was seen in a fibrous stroma with collagen and reticulin fibrils especially peripherally *(Fig 8)*

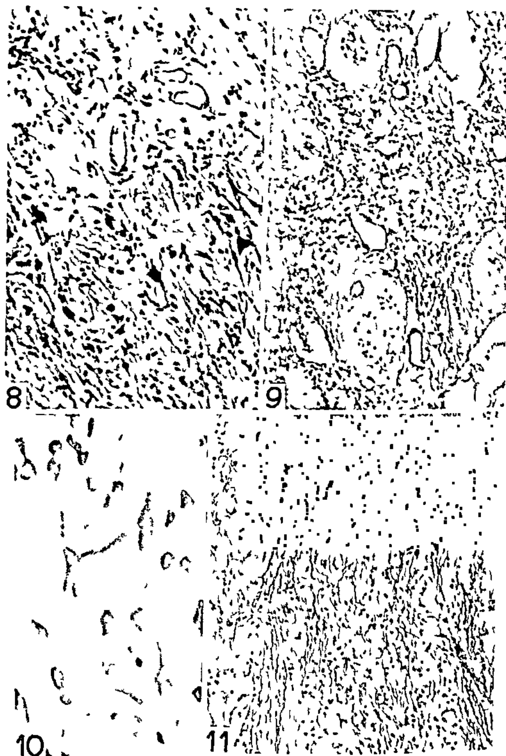
Figs 5 7

Fig 5 A and B Malformed cortical plate with superimposed large tumour like tissue areas in A × 20 which in B are seen to be continuous with the molecular layer Nissl × 100

Fig 6 Cortical area adjacent to tumour like tissue Subpial cell layer left increasing in width merging in *tumour like tissue right Deep portions* of molecular layer relatively poor in cells Nissl × 50

Fig 7 Tumour like tissue composed of cells with oval elongated vesicular and small dark round nuclei Nissl × 1000


5A
5B
6
7



Figs 8 11

Fig 8 Peripheral portions of tumour like tissue with glial cells nerve cells and vessels Nissl × 160
Fig 9 Rounded glial nests in tumour like tissue × 100
Fig 10 Nerve cells in tumour like tissue Nissl × 620
Fig 11 Tumour like tissue with axons partly arranged in fascicles Bodian × 100

where there were a few rounded islands of glial tissue with central glial nuclei surrounded by a fibrillar halo (Fig 9) More mature astrocytes as well as large and small pyramidal nerve-cells (Fig 10) and fusiform or elongated Cajal-cells were also observed Silver impregnation revealed small round cells with scanty cytoplasm and multiple nucleoli and a polar neurite A multitude of frequently myelinated axons ran in different directions or formed slender bundles (Fig 11) Areas rich in collagen and reticulin fibrils showed few axons and *vice versa* No ependymal or choroid plexus formations were seen

were no signs of neoplastic activity These excrescences were lined by a pia like

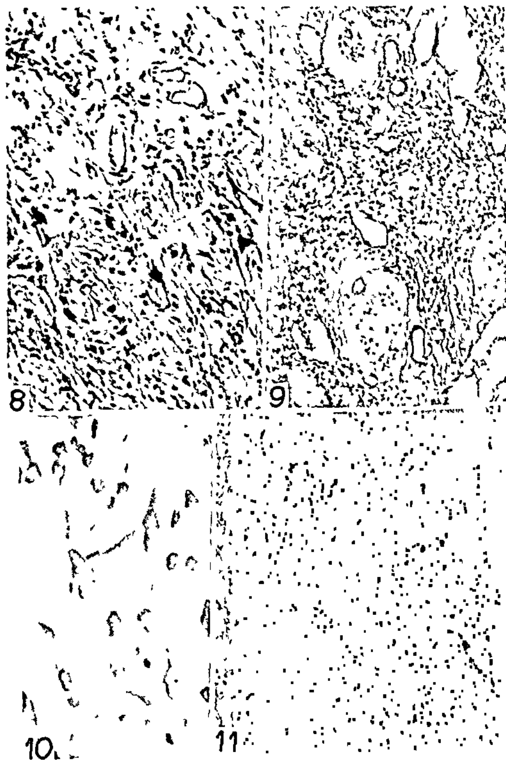
olfactory ventricle surrounded by immature cells arranged in a matrix like fashion The heterotopic islands of grey matter in the centrum semiovale of the central and parietal lobes consisted of well differentiated nerve cells and glial cells. They bulged into the ventricular system covered by ependyma or extended in large conglomerates almost to the basal cortical layers Towards the pori the cortical structures were gradually replaced by a gliovascular tissue the cerebral wall became thinner covered on the outside by a pia, while the inner side which was part of the ventricular wall was devoid of ependymal lining The meninges here showed scattered round cells The membrane covering the right porus consisted of a fibrovascular tissue with only occasional neurites in a ground substance of the type seen within the CNS The cerebellar cortex was largely normal Subcortically one hemisphere showed small islands of grey matter with a micropolygyric pattern In the pons the corticospinal tracts were very slender In the medulla oblongata they were seen as narrow cords of tissue at sites normally occupied by the pyramids The olives and pontine nuclei appeared normal.

DISCUSSION

Autopsy revealed multiple congenital malformations bilateral coloboma oculi, a left vena anonyma emptying into the coronary sinus, microcephaly, biparietal porencephaly and bilateral symmetric areas of cortical malformation with polymicrogyria and a few occasional brain warts Here there were tumour like heterotopias rising from the surface and a subpial granular layer of cells, a constellation suggesting a pathogenetic relationship Other findings included heterotopic grey matter in the centrum semiovale, incompletely differentiated rests of the periventricular matrix, colpocephaly and small areas of micropolygyria in one cerebellar hemisphere The corticospinal tracts from the internal capsule to the medulla oblongata were hypoplastic presumably as a result of the cortical malformation in the central lobes

Before discussing the heterotopias and the subpial granular layer it might be convenient to give a few general remarks on the above-mentioned malformation

Co-existing micropolygyria and porencephaly have been described in a number of cases



Figs 8 11

Fig 8 Peripheral portions of tumour-like tissue with glial cells, nerve cells and vessels Nissl × 160
Fig 9 Rounded glial nests in tumour-like tissue × 100
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Fig 11 Tumour like tissue with axons partly arranged *in fascicles* Bodian × 100

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In addition to these changes 1 2 mm wide mushroom shaped excrescences were seen in the baso temporal fissure, on the hippocampal gyrus as well as on the frontal and occipital lobes (Figs 12 a b and c) Especially the smaller ones seemed to consist entirely of glia while others contained both nerve cells and axons There were no signs of neoplastic activity These excrescences were lined by a pia like membrane which however, seemed to be missing in some areas

[illegible]

poral cortex On its entrance to the brain the olfactory nerve had a persistent olfactory [illegible]

[illegible] The membrane covering the right porus consisted of a fibrovascular tissue with only occasional neurites in a ground substance of the type seen within the CNS The cerebellar cortex was largely normal Subcortically one hemisphere showed small islands of grey matter with a micropolygyric pattern In the pons the corticospinal tracts were very slender In the medulla oblongata they were seen as narrow cords of tissue at sites normally occupied by the pyramids The olives and pontine nuclei appeared normal

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Co-existing micropolygyria and porencephaly have been described in a number of cases


12A


12C


12B

The determination period for micropolygyria falls before the end of the migration period (17) or before the lamination of the cortex is completed i e before the end of the fifth intrauterine month (8)

The presence of heterotopic grey matter in the centrum semiovale indicating retarded migration and areas of micropolygyria with an occasionally rather highly organized cortical plate suggest a determination period between the third and the fifth month of intrauterine life The presence of poorly differentiated or undifferentiated nerve cells and glial cells implies a further component of retarded cellular maturation

I *The Heterotopias*

These can be defined as usually small peninsular or free nests of mainly glial tissue projecting from the surface of the CNS in varying relationship to the meninges They are seen in only 1 per cent of controls but in 25 per cent of patients with malformations of the CNS and are closely related topographically to the malformed parts (10) The heterotopias described in the literature are surveyed in Table 1, from which it is clear that one group of publications (1 8 Table 1) reported cases with supratentorial heterotopias the other (9 18 Table 1) infratentorial whereas a small intermediate group had changes on both sides of the tentorium (9 11 Table 1) The first group included only 8 cases all with solitary or a limited number of heterotopias located supratentorially The heterotopias were small and connected with the cerebral cortex via a stalk They were built up mainly of glial cells and contained only occasional nerve cells but never ependymal or choroid plexus components

The second group consisted of about 100 cases all with multiple changes These were usually small and sometimes had a stalk but they were often embedded in the meninges without any demonstrable relation to the CNS These changes situated around the spinal cord medulla oblongata or pons and mesencephalon consisted of glia occasionally with nerve cells but also of ependymal and choroid plexus formations

The changes in the present case resembled those described in the literature but differed in some respects in spite of their supratentorial location they were multiple and especially the bilateral symmetric changes were unusually large they sprang wide based from the surface of the brain as a continuation of the molecular layer and they contained many nerve cells axons and myelin sheaths

---

Fig 12

A B . . . .
bl.
B

TABLE 1
Reported Marginal Glioneural Heterotopias

	Author		*Localisation*	*Cellular composition*	*Histopathological diagnoses*
1	*Bundschuh*	1912	The dura over the cerebral cortex	Glia	Tuberous sclerosis 1 case
2	Schminke	1920	Over the cerebral cortex	Glia	Megalencephaly 1 case
3	*Buckley & Deery*	1929	Over the cerebral cortex	Glia and astroblasts	Hydrocephalus, 'abnormal cortex" (pachy-micropolygyria * 1 case
4	*Dreller*	1938	Over the cerebral cortex	Glia	Tuberous sclerosis 1 case
5	*Schmidt*	1941	Over the cerebral cortex	Glia + nerve cells	'Hirnwarzen" * 1 case
6	*Crome*	1952	Subdurallly at site of olfactory lobes	Glia	Micropolygyria and arrhinencephaly 1 case
7	*Fischer & Gutmann*	1949	Over the cerebral cortex	Glia nerve cells spongio a neurobl	Micropolygyria, porencephaly, hydrocephalus agnesia of callosal body 1 case
8	*Solcher*	1961	Over the cerebral cortex	Glia	Tuberous sclerosis 1 case
9	*Wohlbach*	1907	Mainly around sp cord, also over the cerebral cortex	Glia, ependyma in 2 heterotopias	Spina bifida, hydrocephalus rhabdomyoma of heart "cortical malformation' 1 case
10	*Cooper & Kernohan*	1951	Infratent approx 96% Over the cerebral cortex approx 4%	Glia in a few ex nerve cells epen dyma granule cells	Malformation of cerebellum, brainstem and cord 70 cases
11	*Abolt & Glass*	1955	Infratent approx 96% Over the cerebral cortex approx 4%	Glia in a few ex nerve cells ependyma, granule cells	Malf of cerebellum, brainstem and cord 30 cases
12	*Oberling*	1924	At mesencephalon	Glia	Hydrocephalus 1 case
13	*Ostertag*	1936	At the post med velum	Glioblasts med blasts, ependyma	Malf of cerebellum, brainstem and cord 2 cases
14	*Ostertag*	1956	Posterior fossa and upper cervical canal	Glia	Malf of cerebellum, brainstem and cord Ependymoma choroid plexus papilloma (survey of lit)
15	*Bailey*	1936	Spinal meninges	Glia	Hydrocephalus Polio Pancreas fibrosis 3 cases
16	*Crome & Mish*	1955	Spinal meninges	Glia	Anancephaly 1 case
17	*Willis*	1962	Spinal meninges	Glia axons nerve cells	No malformation reported 1 case
18	*Popoff & Feigin*	1964	Spinal subarachn space and post fossa	Glia nerve cells axons epend chor plexus	Malformation of cerebellum brainstem and cord 3 cases

* = author's interpretation

The varying names (Table 1) given to these changes may reflect lack of knowledge of their etiology. The names "glial" or "neuroglial" nests seem less appropriate for the changes seen in the present case i.e. lesions consisting of both glial cells and many nerve cells and which have the character of a marginal hyperplasia of heterotopic tissue rather than that of isolated nests. A more suitable name would therefore be *marginal glioneural heterotopias*.

They must be distinguished from the so called *Hirnhernien* and from the brain warts (*Hirnwarzen*).

Hirnhernien, cerebral hernias, consist of brain tissue protruding through the pia or defects in it. They are usually situated on the base of the brain and have the structure of the area from which they emerge sometimes with regressive changes. They have been considered the result of increased intracranial pressure, but also physiological (25, 16). Brain warts (*Hirnwarzen*, 11, 12) are small nodular elevations formed from the second and third cortical layers and projecting into the molecular layer and sometimes giving rise to a small bulge on the pial surface. They are closely related to micropolygyria, in which condition they are also found. On their crowns the molecular layer has been described to contain a layer of glial cells with occasional nerve-cells. In the present case there were a few brain warts but no *Hirnhernien*.

II. *The Subpial Granular Layer*

Ranke (17) described a layer of granular cells appearing in the molecular layer of the cerebral cortex during normal embryogenesis. The present author studied this layer in serial sections of a large number of normal human foetal brains (3). *Ranke*'s designation *die superficielle Kornerschicht*, i.e., the superficial granular layer, is less suitable because the second cortical lamina has been given the same name. The present author has therefore called this layer the foetal subpial granular layer of the cerebral hemispheres, abbreviated SGL.

Judging from personal investigations (3), during the third to fourth months of intrauterine life the SGL first appears in the basal allocortical areas whence it spreads over the cerebral hemispheres, first appearing in the superficial subpial portion of the lamina zonalis separated from the pia by a narrow cell free zone. It becomes widest and densest on the medial and lateral olfactory gyri, where the above-mentioned cell free zone is not seen, but the cells reach or seem to invade the meninges. The SGL is fully developed in the fifth gestational month when it covers the entire surface of the cerebral hemispheres. It then resolves presumably by inward migration, and disappears according to a regional pattern. By birth it has vanished completely except for rests in the prepiriform area and sometimes also in the depth of frontal sulci.

In an unpublished study (4) of the granular cells of the SGL in tissue culture, the cells rapidly differentiated into neuroblasts with long neurites but also into glial cells.

Micropolygyria would seem to be the right setting for the persistence of a SGL because its determination period coincides with, or falls just before, the *period of maximal development of the SGL*. Furthermore, in micropolygyria there is a component of retarded migration which gives rise to heterotopias in the white matter. In the same manner the SGL may be arrested within the molecular layer, with the appearance of a heterotopic structure there.

A subpial layer of nests of glia and nerve cells within the molecular layer has been reported in micropolygyria (22) and in tuberous sclerosis (1, 4, 8) and has been thought to represent remnants of *the superfizielle Kornerschicht* of *Ranke* (17) or SGL. Ranke also suggested the same explanation for the increased density of the cells in the molecular layer in *status verrucosus deformis* of *Ranke* (17), a condition closely related to, or identical with, micropolygyria.

On the basis of these reports and the observation that the cellular layer found in the present case resembles an SGL with respect to the position and cellular composition, it is concluded that we are here dealing with a persistent SGL.

As mentioned in the introduction, some authors postulate the existence of a marginal cellular soil, from which the glioneural heterotopias are thought to spring (6, 23, 7). In the present case such a marginal soil was found in the shape of a persistent SGL. This and the marginal glioneural heterotopias showed the same cellular components, namely, those into which the SGL-cells developed *in vitro*. Both contained cells of embryonal type. A persistent SGL was found in polymicrogyric areas and on the base of the brain, where SGL is best developed during embryogenesis and persists longest and normally tends to involve the meninges. It was in these areas that the heterotopias were found. They emerged *per continuitatem* from the molecular layer *with the persistent SGL*.

The observations set forth above seem to indicate that the marginal glioneural heterotopias take their origin from a persistent SGL.

Work is in progress to test the validity of this assumption.

SUMMARY

Multiple, unusually large and also small marginal glioneural heterotopias were found on the surface of the cerebral hemispheres in a case of micropolygyria. On the basis of literature studies and personal investigations, it is suggested that the marginal glioneural heterotopias derive from the subpial granular layer of the cerebral hemispheres (the *superfizielle Kornerschicht* of *Ranke*) or remnants of this layer present postnatally in certain cortical malformations.

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ATHEROSCLEROSIS IN AN AUTOPSY SERIES

4. *Relation of Aortic Atherosclerosis to Age and Sex*

By

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Received 3 ii 65

Evidence is accumulating that atherosclerosis starts in early childhood (*Geertsen* (5)), but it does not become clinically evident before adult age Thus, it is a slow process, and this is very well illustrated in *Holman, McGill Jr, Strong & Geer*'s (12) concept of the pathogenesis of atherosclerosis They found that every individual above 3 years of age had Sudanophilic intimal deposits in the aorta, and that fibrous plaques appeared about 15 years after the fatty streak Furthermore, they fixed the "clinical horizon", that is the age at which the process gives clinical disease, at about 40 years *A priori* one would expect that such a slowly evolving process should be intimately correlated to time, that is to the age of the individual This is also true Numerous investigations from all over the world unanimously agree that the severity of atherosclerosis rises progressively with age, although at a varying rate in the different countries (*Anderson, Walker, Lutz & Higginson* (1), *Anitschkow* (2), *Bjornsson* (3), *Gore & Hirst Jr* (7), *Gore, Robertson, Hirst, Hadley & Koseki* (8), *Groom, McKee, Webb, Grant, Pean, Hudicourt & Dallemand* (9), *Henschen* (10), *Hirst Jr, Pijaratn & Gore* (11), *Mitchell, Schwartz & Zinger* (15), *Murthy, Dutta & Ramalingaswami* (16), *Sjovall & Wihman* (19), *Spain & Bradess* (21), *Tejada & Gore* (22))

On the basis of twenty reports on atherosclerosis *Anitschkow* (2) concluded that the severity of aortic atherosclerosis in females lagged about 10 years behind that in males in the middle age-groups, and that the sex difference levelled off at about 70 years of age Later investigations have shown similar results The general finding in most series has been a prevalence of atherosclerosis in males in the younger and middle age-groups, but the sex difference disappeared at various age levels, from the fifth to the eighth decade, and thereafter atherosclerosis often prevailed in females (*Bjornsson*'s Copenhagen and Vienna series (3), *Groom, McKee, Webb, Grant, Pean, Hudicourt & Dallemand* (9), *Hirst Jr, Pijaratn & Gore*'s Los Angeles series (11), *Mathur, Patney*

& Kumar (13), *Murthy, Dutta & Ramalingaswami* (16), *Spain & Bradess* (21)) However, some series have not revealed any sex difference at all (*Anderson Walker, Lutz & Higginson* (1), *Hirst Jr, Piyaratn & Gore*s Bangkok series (11), *Meyer, Pepler, Meyer & Theron* (14), *Roberts Jr, Moses & Wilkins* (17), *Roberts Jr, Wilkins & Moses* (18)) Finally, one series showed more atherosclerosis in females than in males (*Bjornsson*'s Iceland series (3)) Recently, *Mitchell, Schwartz & Zinger* (15) confirmed *Anitschkow*s conclusion by stating that females of a given age showed arterial disease of the same severity as males 10 years younger

Thus one may say that in general a definite trend is present in the relation of aortic atherosclerosis to sex, but there are several exceptions and the sex trend does not seem to be as clear and unequivocal as the age trend The exceptions may be due to the smallness of some series Another possibility may be that the selection of the material plays a greater role in the evaluation of the relation of atherosclerosis to sex than to age

MATERIAL

The mater al has been [illegible] ten (4 6)) It consists of 408 [illegible] 15 to 89 years The mean age [illegible] to age and sex will be considered The total cholesterol phosphol poid ratio in the arterial wall—the ChPh value— has been used as index for the severity of atheroscleros s (*Giertsen* (4)) [illegible]

For all indiv dual groups of 6 cases or more the standard error of the mean has been calculated

RESULTS

The Total and Mean Amounts of Atherosclerosis in the Total Series

The total amount of aortic atherosclerosis (the sum total of all ChPh values) was 662 92 (males 346 67 females 316 25) The mean ChPh value was 1 62 in the total series (all ages combined), 1 64 in males and 1 61 in females Thus in this series in which the mean age is identical in the two sexes there the mean amount of aortic atherosclerosis is also identical

The Mean Amount of Atherosclerosis in the Age Sex Groups

Table 1 and Fig 1 show that the mean ChPh value increases almost linearly with age up to the eighth decade in the total series In the last decade there is no further increase

TABLE 1

Aorta. The Mean ChPh-Value and the Standard Error of the Mean within Each Age Group in the two Sexes and in the Total Series

Age group	ChPh value					
	Males		Females		Total	
	Mean	S.E.	Mean	S.E.	Mean	S.E.
15–19	0.48				0.48	
20–29	0.61		0.53		0.57	0.05
30–39	1.01	0.13	0.88	0.07	0.94	0.07
40–49	1.38	0.11	0.98	0.07	1.19	0.07
50–59	1.51	0.09	1.31	0.07	1.42	0.07
60–69	1.76	0.06	1.75	0.06	1.75	0.07
70–79	1.92	0.06	2.0[illegible]	0.0[illegible]	1.99	0.04
80–89	1.98	0.07	1.9[illegible]	0.0[illegible]	1.98	0.05
All age groups	1.64	0.04	1.61	0.04	1.62	0.03

Furthermore, the male value is higher than that of the female in the age-period 20 to 59 years. In the next two decades the female value increases more rapidly. Consequently, the male and female values are identical in the seventh decade, and the female value is highest in the eighth decade. In the ninth decade they are again identical. It should be noted that the male values in the thirties and the fourties correspond to values in females about 10 years older. However, the sex difference is significant only in the 40–49 year age-group.

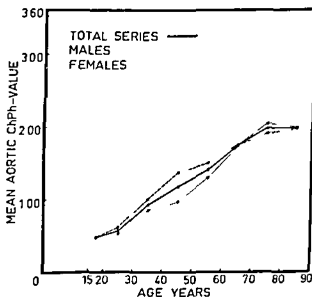


Fig. 1

Aortic atherosclerosis. The variation of the mean ChPh value within the age groups in the total series and in each sex.

TABLF 2

Aorta The Number of Cases the Mean Age and the Stan lard Error of the Mean within Specified ChPh Groups in the two Sexes and in the Total Series

ChPh gr up	Age Males No of cases	Mean	S F	Females No of cases	Mean	S E.	Total No of cases	Mean	S E.
0 00 0 19									
0 20-0 39				1	26 0		1	26 0	
0 40 0 59	5	22 0		4	28 8		9	25 0	2 6
0 60 0 79	16	38.3	3 6	22	40 0	2 3	38	39.3	2 0
0 80-0 99	14	50 6	2 7	11	45 5	1 7	25	48 4	1 7
1 00 1 19	18	57 3	2 6	14	53 6	2 7	32	55 7	1 9
1 20-1 39	15	57 7	3 5	18	56.3	2 6	33	56 9	2 1
1 40 1 59	24	67.3	2 3	21	66 5	2 1	45	66 9	1 6
1 60 1 79	27	61 7	2 0	26	67 2	2 3	53	64 4	1 5
1 80-1 99	25	65 4	2 1	27	70 0	1 5	52	67 6	1 3
2 00 2 19	33	74 0	1 9	15	69 9	1 7	48	72 7	1 5
2 20 2.39	21	71 1	2 1	22	76 2	1 7	43	73 7	1.3
2 40 2 59	7	67 0	6 9	8	74 8	2 3	15	71 1	3 5
2 60 2 79	4	72 3		8	75 0	1 6	12	74 1	1 7
2 80 2 99	1	57 0					1	57 0	
3 00 3 19									
3 20 3 39									
3 40-3 59	1	65 0					1	65 0	

The Mean Age at Specified Amounts of Atherosclerosis

Table 2 and Fig 2 show the case distribution, the mean age, and the standard error of the mean within specified ChPh groups The mean age is 25 years in the first well represented group in the total series

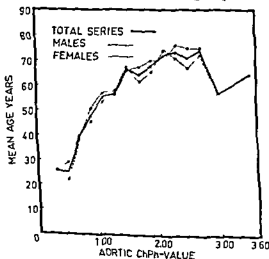


Fig 2

Aort c atherosclerosis The variation of the mean age in the ChPh groups in the total series and in each sex

(the 0 40–0 59 group) Subsequently, the mean increases with the ChPh value until the 2 00–2 19 group, when it is 72 2 years After that, the age is approximately constant

Moreover, the mean age is 6 8 years lower in males than in females in the 0 40–0 59 ChPh-group The difference is smaller in the next group, and after that, there is obviously no definite, consistent sex difference

The Correlation between Age and Atherosclerosis

Table 3 shows the correlation between age and ChPh-value The correlation coefficients were +0 57 in males, +0 75 in females, and +0 66 in the total series The difference between the z-transformed male and

TABLE 3
Aorta The Correlation between the ChPh-Value and Age in the two Sexes and in the Total Series

Age	0 00–0 19	0 20–0 39	0 40–0 59	0 60–0 79	0 80–0 99	1 00–1 19	1 20–1 39	1 40–1 59	1 60–1 79	1 80–1 99	2 00–2 19	2 20–2 39	2 40–2 59	2 60–2 79	2 80–2 99	3 00–3 19	3 20–3 39	3 40–3 59
									ChPh value									
									Males									
15–19			3															
20 29			1	3														
30 39			1	8	3	1	1		1	1			1					
40–49				2	2	1	6	2	1	2	2							
50 59				2	8	5	1	3	7	3	3	3	1		1			1
60 69					1	7	3	7	15	11	4	6	1	1				
70–79				1		1	4	9	2	6	11	10	2	3				
80 89						1	1	3	1	2	13	2	2					
									Correlation coefficient r = +0 57									
									Females									
15–19																		
20–29		1	2	2														
30 39			2	10	3	1	2		1									
40–49				6	4	1	2	1	1									
50 59				3	4	6	7	4	3	2	2							
60 69				1		3	5	8	8	12	4	4	2	1				
70–79						1	1	4	9	10	8	11	3	6				
80 89							1	4	4	3	1	7	3	1				
									Correlation coefficient r = +0 75									
									Total series									
15 19			3															
20 29		1	3	5														
30 39			3	18	6	2	3		2	1			1					
40–49				8	[illegible]	6	7	3	2	2	2							
50–59				5	12	11	8	7	10	5	5	3	1		1			1
60–69				1	1	10	8	15	23	23	8	10	3	2				
70–79				1		2	5	13	11	16	19	21	5	9				
80 89						1	2	7	5	5	14	9	5	1				
									Correlation coefficient r = +0 66									

female coefficients is significant (*Snedecor* (20)) Thus there is a fairly strong correlation between age and aortic atherosclerosis stronger in females than in males

The Significance of Age and Sex for the Inter Relationship between Atherosclerosis in Different Arteries

Table 4 shows for each aortic ChPh group of 15 or more cases the correlation between the corresponding ChPh values and age in the coronary and the cerebral arteries It appears that the correlation coefficient shows a great variation from group to group in both arteries It is sometimes negative sometimes positive without any general trend Obviously age is of no significance in the inter relationship No subdivision into sex groups has been made because the groups would be numerically small

TABLE 4

The Correlation between Age and the Coronary and Cerebral ChPh Values which Correspond to Specified Aortic Values

Aorta		Correlation ChPh value age	
ChPh value	No of cases	Coronary arteries r	Cerebral arteries r
0.60–0.79	38	+0.31	−0.14
0.80–0.99	25	+0.38	+0.35
1.00–1.19	32	−0.07	−0.20
1.20–1.39	33	−0.18	+0.16
1.40–1.59	45	+0.27	+0.19
1.60–1.79	53	+0.10	+0.31
1.80–1.99	52	−0.02	+0.21
2.00–2.19	48	−0.18	+0.02
2.20–2.39	43	+0.3	+0.12
2.40–2.59	15	−0.43	−0.16

However the mean coronary and cerebral ChPh values which correspond to a specified aortic value have been calculated for each sex The values for the total series have been previously examined (*Giertsen* (6)) Table 5 and Fig 3 show that the values for the two sexes follow each other very closely with increasing aortic value both in the coronary and the cerebral arteries The cerebral values are somewhat irregular at aortic values above 2.00 but as a whole no sex prevails systematically over the other

Finally the correlation between the corresponding ChPh values in the aorta and coronary arteries and in the aorta and cerebral arteries has been calculated for each sex The coefficients for the former correlation were +0.60 for males and +0.69 for females and for the latter +0.56 for males and +0.60 for females The difference between the z transformed coefficients is not significant for any of the correlations The correlation coefficients for the total series have been previously examined (*Giertsen* (6))

TABLE 5

The Aortic ChPh Value Compared with the Corresponding Mean Coronary and Cerebral Values in the two Sexes (The Case Distribution Is Given in Table 2)

Aorta	ChPh value							
	Coronary arteries				Cerebral arteries			
	Males		Females		Males		Females	
	Mean	S E	Mean	S E	Mean	S E	Mean	S E
0.00 0.19								
0.20 0.39			0.68				0.40	
0.40 0.59	*0.57*		*0.59*		*0.43*		*0.45*	
0.60 0.79	1.08	0.13	0.75	0.08	0.46	0.05	0.44	0.04
0.80 0.99	1.43	0.12	1.17	0.18	0.55	0.08	0.56	0.11
1.00 1.19	1.70	0.10	1.78	0.18	0.67	0.09	0.66	0.09
1.20 1.39	1.72	0.11	1.54	0.11	0.74	0.10	0.67	0.08
1.40 1.59	*1.71*	*0.10*	*1.69*	*0.11*	*0.98*	*0.10*	*0.89*	*0.12*
1.60 1.79	1.97	0.09	1.97	0.10	1.09	0.11	1.19	0.12
1.80 1.99	2.09	0.08	1.98	0.08	1.28	0.14	1.10	0.13
2.00 2.19	2.07	0.06	2.24	0.10	1.60	0.13	1.51	0.20
2.20 2.39	2.19	0.07	2.38	0.08	1.48	0.17	2.01	0.18
2.40 2.59	2.27	0.19	2.37	0.06	1.34	0.27	2.12	0.19
2.60 2.79	2.21		2.03	0.22	2.12		1.24	0.31
2.80–2.99	2.53				3.10			
3.00 3.19								
3.20 3.39								
3.40 3.59	2.04				1.43			

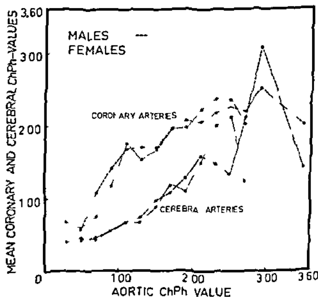


Fig 3

Aortic atherosclerosis The variation of the mean coronary and cerebral ChPh values which correspond to specified aortic values in each sex

DISCUSSION

The age and sex trend of aortic atherosclerosis in this series is by and large very similar to that in the majority of the previously cited series On an average, the severity of atherosclerosis rose practically linearly with age from 15 to 79 years in the total series One should, however, be aware of the great individual variations.

As would be expected, the correlation between age and atherosclerosis was fairly strong, but it is perhaps surprising that it was stronger in females than in males No explanation can be offered for this, except the possibility that it may be a coincidence, a peculiarity of the series

It has previously been found that the ChPh-values in the first three 5 year-periods in children were 0 33, 0 39 and 0 49 (*Giertsen* (5)) The last value is identical to that in the 15–19 year age-group of the present series Admittedly, the number of cases in this group was small, but the figures indicate an even progress of the atherosclerotic process from childhood to adult age This seems to contradict the finding of *Holman, McGill Jr, Strong & Geer* (12) that atherosclerosis—as judged by the amount of intimal fatty streaks—rose precipitously in Negroes from the second to the third, and in whites from the third to the fourth 5 year-period The discrepancy may, however, be due to the different grading methods used Discrepancies which arise when results, based on gross grading and on chemical grading, are compared, have been previously discussed (*Giertsen* (4, 6)) Suffice it to say that the aorta may or may not display visible changes within the ChPh-interval 0 38–0 51 Therefore, when a gross grading system is employed, an artery without visible changes will be classified as normal, although it may have reached chemically the same stage of atherosclerosis as an artery in which a few fatty streaks are found

ChPh values below the interval mentioned invariably denoted grossly normal arteries, whereas values above the interval always denoted visible changes From this, and from Tables 1 and 3, follow that the mean ChPh-value indicates visible changes already in the twenties, but not until the fourties do all the aortas show a ChPh-value above the interval, that is display visible changes In fact, only 2 aortas in this series were grossly normal (a 22-year old male, and a 32 year-old female)

Unfortunately, the present series does not contain any females in the 15 19 year age group However, the sex difference is very small in the twenties and thereafter it increases gradually till the fourties This pattern suggests that only a very small, if any, sex difference could have been present in the 15–19 year age group It is tempting to [illegible] this [illegible]

[illegible] ay

[illegible] to

[illegible] on one single factor

In the present series, the mean amount of aortic atherosclerosis in

females lagged about 10 years behind that in males in the middle life, and the sex difference disappeared in the seventh decade In other series it disappeared in the fifth (*Groom, McKee, Webb, Grant, Pean, Hudicourt & Dallemand* (9)), the sixth and seventh (*Anitschkow* (2)), and the eighth (*Henschen* (10)) decade Thereafter, females usually showed more atherosclerosis than males It seems difficult to link this reversion of the sex ratio to the onset of the menopause If this were of a major significance, a more uniform pattern would probably have been expected in the various series

The selection of the series must also be taken into consideration The factors which accelerate and retard the atherosclerotic process may be unevenly distributed within the two sexes If so, the selection may produce or obscure a sex difference It is true that most series show a sex difference, but, as mentioned in the introduction, several do not, and *Roberts Jr*, *Moses & Wilkins* (17) suggested that vascular catastrophes might be sex related, but not necessarily the extent and severity of atherosclerosis

It has previously been shown that there is a strong correlation between atherosclerosis in the aorta, the coronary and the cerebral arteries (*Giertsen* (6)) There were, however, wide individual variations This could be the result of the influence of age and/or sex Thus, in a specified aortic ChPh-group the corresponding coronary and cerebral values might vary with these factors However, our findings denote that neither age nor sex is of significance in the inter-relationship, when the aorta is taken as basis for the comparison In other words, at any given aortic ChPh-value the corresponding coronary and cerebral values are independent of the individual's age and sex

All in all, it may be said that the age trend of aortic atherosclerosis is well established, but the sex trend is not quite clear

SUMMARY

The relation of aortic atherosclerosis to age and sex has been examined in 408 individuals, 211 males and 197 females, ranging in age from 15 to 89 years, and with a mean age of 62 3 years in males and 62 9 years in females The total cholesterol/phospholipoid ratio in the arterial wall—the ChPh-value—has been used as index for the degree of atherosclerosis present

Atherosclerosis was found to rise progressively, and practically linearly, with age until the eighth decade No further increase occurred in the ninth decade The correlation to age was fairly strong, stronger in females than in males There were, however, very wide individual variations

In the age-period from 20 to 59 years males showed more atherosclerosis than females, but the difference was significant only in the 40–49 year age-group In the eighth decade females showed more atheroscle-

rosis than males In the total series (all ages combined) the mean amount of atherosclerosis was identical in the two sexes

In specified ChPh groups, that is at specified amounts of aortic atherosclerosis there was no consistent difference between the mean age in the two sexes

The inter relationship between atherosclerosis in the aorta and the coronary arteries, and in the aorta and the cerebral arteries, is not influenced by the age or the sex of the individual, when the aorta is taken as basis for the comparison

It is stressed that the selection of the series may have a decisive influence on the sex trend

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ATHEROSCLEROSIS IN AN AUTOPSY SERIES

5 *Relation of Coronary Atherosclerosis to Age and Sex*

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Received 3 ix 65

As a rule fatty streaks appear in the aorta already in the first decade (*Holman McGill Jr Strong & Geer* (13)) In the coronary arteries they are exceptional in the first but appear regularly in the second decade (*Anitschkow* (2) *Bahr* (3) *Strong & McGill Jr* (20), *Wolkoff* (22)) All observations on coronary atherosclerosis agree that it is intimately related to age Thus in *Henschen*s (11) series there was an almost linear progress with age through all age groups to the ninth decade The same was true in the series of *Gore & Hirst Jr* (8), *Gore, Robertson Hirst, Hadley & Koseki* (9), and *Lober* (14), but in these series the severity of atherosclerosis declined after the eighth decade In the series of *Groom McKee, Webb, Grant, Pean, Hudicourt & Dallemand* (10) the increase was fairly sharp until the fifth decade, but very slight in the subsequent decades The opposite was true in the series of *Hirst Jr Piyaratn & Gore* (12) and *Mathur, Patney & Kumar* (15) with only a slight increase until the fourth decade and thereafter a sharp rise Thus there seems to be no general age trend common to all series but it is obvious that the question is not whether or not—only how—the process is related to age

Practically all series show a sex difference in coronary atherosclerosis and it is variously stated that females lag from 5 to 20 years behind males in the severity of the process *Lober* (14) found a sex difference already in the first decade and *Strong & McGill Jr* (20) in the second In Lober s series the difference persisted through all the age groups The same was true in the series of *Bahr* (3), *Henschen* (11) and *Mathur Patney & Kumar* (15) However, *Strong & McGill Jr* (20) found a sex difference only in the younger age groups and only in whites, not in Negroes In the series of *Meyer, Pepler, Meyer & Theron* (16) a sex difference was present only in the fifth decade in the white subjects and only in the sixth decade in the Bantu *Groom, McKee, Webb, Grant Pean Hudicourt & Dallemand* (10) found more atherosclerosis in males than in females up to the age of fifty years Thereafter, no further increase occurred in the males whereas the process continued

to advance in females, and surpassed that in males in the older age groups. A similar trend in males was found by *White, Edwards & Dry* (21), and in females by *Ackerman, Dry & Edwards* (1). Finally, in a series of patients dying without vascular catastrophes a sex difference was present only in the fourties (*Roberts Jr., Moses & Wilkins* (17)), and in a series with vascular catastrophes no sex difference was found at all (*Roberts Jr., Wilkins & Moses* (18)). Thus, it may be said that a sex difference has usually been found up to about 50 years of age, but after that the findings differ from series to series.

When the varying age and sex trends in the different reports are considered, it should be borne in mind that the results are based on autopsy materials from widely different sources, and that many different grading systems have been used to measure the severity of atherosclerosis. This makes a comparison of the results very difficult.

MATERIAL

The material has been previously described (*Geertsen* (4, 6)). It consists of 408 individuals, 211 males and 197 females, ranging in age from 15 to 83 years. The mean age was 62.3 years in males and 62.9 in females.

In this paper the relation of coronary atherosclerosis to age and sex will be considered. The total cholesterol/phospholipoid ratio in the arterial wall—the ChPh value—has been used as index for the severity of atherosclerosis (*Geertsen* (4)).

The mean ChPh value has been calculated for each age and sex group, and the mean age for each sex and ChPh group. Furthermore, the correlation between age and ChPh value has been examined in both sexes and in the total series. Finally, the significance of age and sex for the inter-relationship between atherosclerosis in the aorta, the coronary and the cerebral arteries has been examined.

For all individual groups of 6 cases or more the standard error of the mean has been calculated.

RESULTS

The Total and Mean Amounts of Atherosclerosis in the Total Series

The total amount of coronary atherosclerosis (the sum total of all ChPh-values) was 732.47 (males 387.06, females 345.41). The mean in the total series (all ages combined) was 1.79, 1.83 in males and 1.75 in females. The sex difference is not significant. Thus, in this series, in which the mean age is identical in the two sexes, there the mean amount of coronary atherosclerosis is also practically identical.

The Mean Amount of Atherosclerosis in the Age-Sex Groups

Table 1 and Fig. 1 show that the mean ChPh value increases rapidly with age until the seventh decade. In the last two decades the increase is only slight. The male value shows a very rapid rise until the 40–49 year age-group. In the next age-group it decreases slightly, but thereafter it rises again. The female value is practically identical to that of the male in the 20–29 year age-group. The increase with age is comparatively slow until the fourties, but subsequently it is more rapid

and becomes higher than the male value in the last two decades It appears that the values for males in the thirties and fourties are reached by females almost 20 years later The sex difference is fairly large and significant in the two decades from 30 to 49 years The differences in the last two decades are not significant

The Mean Age at Specified Amounts of Atherosclerosis

In the total series the mean age is 30 5 years in the first well represented group (the 0 20 0 39 group) Subsequently, the age increases re

TABLE 1

Coronary Arteries The Mean ChPh Value and the Standard Error of the Mean within each Age Group in the two Sexes and in the Total Series

Age group	ChPh value					
	Males		Females		Total	
	Mean	S E.	Mean	S E.	Mean	S E
15 19	0 45				0 45	
20 29	0 65		0 63		0 64	0 08
30-39	1 53	0 21	0 94	0 14	1 22	0 13
40-49	1 81	0 15	1 00	0 14	1 43	0 12
50-59	1 69	0 09	1 64	0 13	1 67	0 07
60 69	1 97	0 05	1 93	0 06	1 95	0 04
70-79	1 98	0 07	2 09	0 06	2 04	0 05
80-89	2 03	0 07	2 19	0 09	2 11	0 06
All age groups	1 83	0 04	1 75	0 05	1 79	0 03

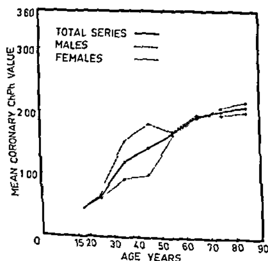


Fig 1

Coronary atherosclerosis The variation of the mean ChPh value within the age groups in the total series and in each sex

gularly with the ChPh-value until the 1 40–1 59 group With a further increase in the ChPh-value the age varies only within narrow limits (Table 2, Fig 2)

TABLE 2

Coronary Arteries The Number of Cases the Mean Age and the Standard Error of the Mean within Specified ChPh Groups in the two Series and in the Total Series

ChPh group	Age								
	Males			Females			Total		
	No of cases	Mean	S E	No of cases	Mean	S E	No of cases	Mean	S E
0 00–0 19				1	39 0		1	39 0	
0 20 0 39	2	16 5		4	37 5		6	30 5	4 7
0 40 0 59	7	31 8	3 1	8	38 0	4 6	15	35 1	2 9
0 60 0 79	4	40 0		9	45 3	3 6	13	43 7	3 4
0 80 0 99	5	56 2		10	41 5	3 8	15	46 4	3 6
1 00 1 19	11	52 2	5 0	12	50 5	4 1	23	51 3	3 1
1 20 1 39	15	49 8	2 7	17	56 9	3 6	32	53 6	2 4
1 40–1 59	15	67 7	3 4	11	62 8	3 6	26	65 6	2 4
1 60 1 79	27	62 9	2 6	15	66 5	2 5	42	64 2	1 9
1 80 1 99	38	67 9	1 9	21	67 1	2 4	59	67 6	1 5
2 00 2 19	35	69 0	1 6	32	70 1	1 8	67	69 5	1 2
2 20 2 39	22	66 7	2 6	26	72 3	1 4	48	69 8	1 5
2 40 2 59	18	62 9	3 4	14	69 5	2 6	32	65 8	2 3
2 60–2 79	5	62 0		11	68 1	5 5	16	66 2	4 3
2 80 2 99	4	61 5		4	61 8		8	61 6	6 0
3 00–3 19				1	80 0		1	80 0	
3 20 3 39	3	61 0					3	61 0	
3 40 3 59				1	72 0		1	72 0	

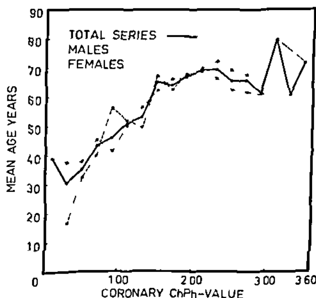


Fig 2

Coronary atherosclerosis The variation of the mean age in the ChPh groups in the total series and in each sex

Furthermore, it appears that the mean age is 21 years lower in males than in females in the ChPh group 0 20 0 39 However, this group is poorly represented In the next two groups the age is about 6 and 5 years lower in males than in females Subsequently, the age in males is soon lower and soon higher than in females The sex difference is not significant in any ChPh group

The Correlation between Age and Atherosclerosis

Table 3 shows the correlation between age and atherosclerosis in the total series and in the two sexes In the former the correlation coefficient is +0 52 in males +0 40 and in females +0 63 The difference between the male and female z transformed coefficients is signi

TABLE 3

Coronary Arteries The Correlation between the ChPh Values and Age in the two Sexes and in the Total Series

Age	ChPh value 0 00 0 19	0 20 0 39	0 40 0 59	0 60 0 79	0 80 0 99	1 00 1 19	1 20 1 39	1 40 1 59	1 60 1 79	1 80 1 99	2 00 2 19	2 20 2 39	2 40 2 59	2 60 2 79	2 80 2 99	3 00 3 19	3 20 3 39	3 40 3 59
Males																		
15 19		2	1															
20-29			2	1		1												
30-39			3	1	1	2	3		2	1			1	1	1		1	
40 49			1	1			4	1	3	2		2	4		1			
50-59				1	2	4	6	3	5	3	5	4	3	1				
60 69					1	2	1	3	8	14	14	7	5	2				
70-79					1	2	1	5	5	12	11	6	3		1		2	
80-89								3	4	6	5	3	2	1	1			
Correlation coefficient r = +0 40																		
Females																		
15 19																		
20 29			2	1	1	1												
30-39	1	2	3	2	4	2	3			1				1				
40-49		2	1	3	2	4	2	2						1				
50-59			2	2	3	1	4	2	4	2	5		3	1	2			
60-69				1		3	3	3	4	10	9	12	1	1	1			
70 79						1	4	3	6	5	11	9	8	4	1			1
80 89							1	1	1	3	7	5	2	3		1		
Correlation coefficient r = +0 63																		
Total series																		
15 19		2	1															
20-29			4	2	1	2												
30-39	1	2	6	3	5	4	6		2	2			1	2	1		1	
40 49		2	2	4	2	4	6	3	3	2		2	4	1	1			
50 59			2	3	5	5	10	5	9	5	10	4	6	2	2			
60 69				1	1	5	4	6	12	24	23	19	6	3	1			
70-79					1	3	5	8	11	17	22	15	11	4	2		2	1
80 89							1	4	5	9	12	8		4	1	1		
Correlation coefficient r = +0 52																		

ficant (Snedecor (19)). Thus, there is a fairly strong, positive correlation between age and coronary atherosclerosis, stronger in females than in males.

The Significance of Age and Sex for the Inter-Relationship between Atherosclerosis in the Different Arteries

Table 4 shows for each coronary ChPh-group of 15 cases or more the correlation between age and the corresponding aortic and cerebral values. It appears that the coefficients for the correlation age-aortic values are positive, and fairly high in all the coronary ChPh-groups. The coefficients are significant, except in the first group. The first two coefficients for the correlation age-cerebral values are negative, while all the others are positive. However, they vary much from group to group, and some are fairly high, but only three are significant (ChPh groups 1.40–1.59, 1.60–1.79, 2.00–2.19). No subdivision into sex-groups has been made, as the groups would be numerically small.

TABLE 4

The Correlation between Age and the Aortic and Cerebral ChPh Values which Correspond to Specified Coronary Values

Coronary arteries		Correlation ChPh value age	
ChPh value	No of cases	Aorta r	Cerebral arteries r
0.40–0.59	15	+0.36	0.30
0.60–0.79	13	+0.75	−0.18
0.80–0.99	15	+0.59	+0.12
1.00–1.19	23	+0.61	+0.35
1.20–1.39	32	+0.41	+0.08
1.40–1.59	26	+0.46	+0.62
1.60–1.79	42	+0.75	+0.42
1.80–1.99	59	+0.45	+0.15
2.00–2.19	67	+0.32	+0.44
2.20–2.39	48	+0.30	+0.06
2.40–2.59	32	+0.38	+0.33
2.60–2.79	16	+0.74	+0.19

Table 5 and Fig. 3 show the aortic and cerebral ChPh-values which correspond to specified coronary values in each sex. It appears that the male and female values follow each other very closely with increasing coronary values in both arteries, and no sex prevails systematically over the other.

In a previous paper no significant sex difference was found in the correlation between aortic and coronary atherosclerosis (*Gertsen* (7)). The coefficient for the correlation between coronary and cerebral atherosclerosis is +0.40 in males and +0.59 in females. The difference between the z-transformed coefficients is significant.

TABLE 5

The Coronary ChPh Value Compared with the Corresponding Mean Aortic and Cerebral Values in the two Sexes (The Case Distribution is Given in Table 2)

Coronary arteries	ChPh value Aorta				Cerebral arteries			
	Males		Females		Males		Females	
	Mean	S E	Mean	S E	Mean	S E.	Mean	S E.
0 00–0 19			0 75				0 09	
0 20–0 39	0 48		0 65		0 32		0 18	
0 40–0 59	0 76	0 13	0 68	0 05	0 43	0 06	0 53	0 13
0 60–0 79	0 78		0 91	0 11	0 52		0 43	0 04
0 80–0 99	1 02		0 89	0 08	0 51		0 60	0 05
1 00–1 19	1 10	0 13	1 26	0 13	0 80	0 17	0 53	0 09
1 20 1 39	1 20	0 11	1 43	0 14	0 71	0 14	0 69	0 09
1 40–1 59	1 37	0 12	1 46	0 10	0 88	0 10	0.83	0 11
1 60 1 79	1 68	0 09	1 68	0 14	0 97	0 12	0 75	0 14
1 80 1 99	1 72	0 07	1 66	0 07	1 10	0 09	1 01	0 16
2 00–2 19	1 91	0 09	1 86	0 06	1.28	0 13	1 44	0 13
2 20–2 39	2 07	0 09	2 07	0 07	1 61	0 15	1 51	0 12
2 40–2 59	1 92	0 09	1 92	0 13	1 54	0 20	1 73	0 21
2 60 2 79	2 00		2 06	0 11	0 91		1.32	0 23
2 80–2 99	2 25		1 93		1 60		1 78	
3 00 3 19			2 22				3 59	
3 20–3 39	1 86				1 19			
3 40–3 59			2 27				3 19	

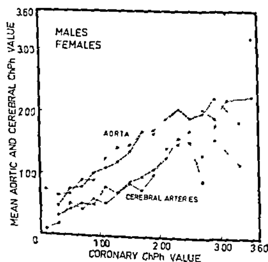


Fig 3

Coronary atherosclerosis The variation of the mean aortic and cerebral ChPh values which correspond to specified coronary values in each sex

DISCUSSION

Our findings confirm that coronary atherosclerosis is intimately correlated to age On an average, the degree of atherosclerosis increases considerably through the age-groups

In the total series the coefficient for the correlation age coronary atherosclerosis, +0 52, is lower than that previously found for the correlation age-aortic atherosclerosis, *viz* 0 66 (*Giertsen* (7)) The difference between the *z*-transformed coefficients is significant Thus, the correlation between age and atherosclerosis is weaker in the coronary arteries than in the aorta The correlation between age and atherosclerosis was found to be stronger in females than in males, just as in the aorta However, the correlation tables show very great individual variations in the age-sex groups, and an individual of 70 years may have just as little coronary atherosclerosis as one of 20 years

It has previously been shown that the coronary arteries may or may not display visible changes within the ChPh-interval 0 37–1 00 (*Giertsen* (4)) This range is considerably greater than that in the aorta, *viz* 0 38–0 51 This indicates that the coronary arteries can tolerate a higher ChPh-value than the aorta and still appear grossly normal Table 1 indicates that visible changes will, on an average, be present after 30 years in males, and after 40 years in females This is 10 and 20 years later than in the aorta (*Giertsen* (7)) Table 3 shows that even 60- and 70-year-old individuals may have a coronary ChPh-value below 1 00, which denotes that their coronary arteries may look normal or display only minimal gross changes However, in this series, all individuals above 60 years showed more or less visible changes

Our findings indicate that the development of atherosclerosis in females parallels that in males in middle life but lags about 20 years behind This sex difference is somewhat greater than that found in aortic atherosclerosis, *viz* 10 years (*Giertsen* (7)) However, the male curve is uneven, and it seems unreasonable that males should have less coronary atherosclerosis in the fifties than in the fourties Thus, the male curve does probably not reflect the true course of the development of atherosclerosis Either do the males in this series have an exceptionally little advanced atherosclerosis in the fifties, or an exceptionally far advanced atherosclerosis in the fourties, and possibly also in the thirties In other words, the course of the curve may be a product of the selection of the material

It has previously been shown that there is a strong correlation between atherosclerosis in the aorta, the coronary and the cerebral arteries (*Giertsen* (6)) However, there were great individual variations This might be due to the influence of age and or sex The coronary and cerebral values which correspond to specified aortic values are, however, independent of age and sex (*Giertsen* (7)) In this paper it has been found that the aortic, but not the cerebral, values which corres-

pond to specified coronary values, are related to age None of them are related to sex Therefore, some of the variations in the inter relationship between atherosclerosis in the aorta and the coronary arteries may be due to the influence of age This must possibly be seen in relation to the stronger correlation between age and atherosclerosis in the aorta than in the coronary arteries Finally, the correlation between coronary and cerebral atherosclerosis is slightly stronger in females than in males

All in all, it is obvious that the sex trend in coronary atherosclerosis is not quite clear, and that further studies are needed before definite conclusions can be drawn

SUMMARY

The relation of coronary atherosclerosis to age and sex has been examined in 408 individuals, 211 males and 197 females, ranging in age from 15 to 89 years and with a mean age of 62 3 years in males and 62 9 in females The total cholesterol phospholipoid ratio in the arterial wall—the ChPh value—has been used as index for the degree of atherosclerosis

Atherosclerosis was found to rise progressively with age, and the correlation between age and atherosclerosis was fairly strong, stronger in [illegible] wever, great individual variations [illegible] in the two sexes (all ages com [illegible]

Females lagged almost 20 years behind males in the severity of atherosclerosis in middle life In the eighth and ninth decade they showed more atherosclerosis than males The sex difference was significant only in the 30–39 and 40–49 year age groups However, the values for males did probably not reflect the true course of atherosclerosis in this sex

At specified amounts of coronary atherosclerosis there was no definite consistent difference in age between the two sexes, except for a lower age in males than in females at the lowermost ChPh values

Furthermore, at specified amounts of coronary atherosclerosis the corresponding amounts of aortic atherosclerosis increased with age, but not that of cerebral atherosclerosis There was no sex difference in the inter relationship between coronary and aortic atherosclerosis, but the correlation between coronary and cerebral atherosclerosis was stronger in females than in males

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Atherosclerosis was found to rise progressively with age, and the correlation between age and atherosclerosis was fairly strong stronger in females than in males There were, however, great individual variations The mean amount of atherosclerosis in the two sexes (all ages combined) was identical

Females lagged almost 20 years behind males in the severity of atherosclerosis in middle life In the eighth and ninth decade they showed more atherosclerosis than males The sex difference was significant only in the 30 39 and 40–49 year age groups However, the values for males did probably not reflect the true course of atherosclerosis in this sex

At specified amounts of coronary atherosclerosis there was no definite consistent difference in age between the two sexes except for a lower age in males than in females at the lowermost ChPh values

Furthermore at specified amounts of coronary atherosclerosis the corresponding amounts of aortic atherosclerosis increased with age, but not that of cerebral atherosclerosis There was no sex difference in the inter relationship between coronary and aortic atherosclerosis but the correlation between coronary and cerebral atherosclerosis was stronger in females than in males

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ON THE INHERITANCE OF THE KELL BLOOD GROUP SYSTEM

A Study of One Hundred Families

By

AAGE HEIKEN

Received 27 ii 65

Anti K (Kell) discovered by *Coombs et al* (4) as well as anti k (Cellano) discovered by *Levine et al* (9) occur as iso immune antibodies in human sera According to the genetic interpretation of the Kell blood group system the corresponding antigens K and k are determined by a pair of autosomal alleles, which give rise to three demonstrable genotypes KK Kk and kk (11)

In recent years however, a number of observations have shown that the Kell system is more complex than was at first believed Two antigens Kpᵃ (1) and Kpᵇ (2) have been detected These antigens are antithetic and probably produced by two allelic genes linked to the K k locus Several examples of blood lacking not only K and k but also Kpᵃ and Kpᵇ antigens have been met with (3 8 see also 11) In addition *Corcoran et al* (5) have found an antibody (anti Ku) revealing an antigen of high frequency related to the Kell system Anti Ku was produced by a woman of the unusual K— k— Kp(a —b—) type mentioned above

Owing probably to the scarcity of anti k sera no unselected families have as far as is known been tested with both anti K and anti k Comprehensive family investigations have been carried out with anti K but due to the low frequency of the K gene the number of K+ × K+ and K+ × K— matings are relatively limited Among 460 English families with 1 022 children (11) and 184 Danish families with 437 children (6 10) only 9 K+ × K+ and 93 K+ × K— matings were obtained It was therefore considered of interest to report the results of the present investigation

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the observed number of k— children was a little higher than expected, resulting in a rather high χ^2 value, but the difference between observed and expected recessives was insignificant

TABLE 3

Mating Type K+ × K—

Analysis of Families with and without Children of Group k—

No of children in family	Total no of families	No of families with at least one k child		
		Obs	Exp	Variance
1	1	0	0 491	0 250
2	5	4	3 680	0 971
3	4	4	3 436	0 485
4	1	0	0 920	0 073
Total	11	8	8 527	1 779

$\chi^2 = 0.527^2 / 1.779 = 0.156$ d f = 1 0 70>P>0 50

The expected number of families with at least one k— child, recorded in Table 3 was calculated on the basis of the results of a population investigation carried out by *Heiken* (7) This investigation included 4 527 unrelated Swedish children and the following phenotype gene and genotype frequencies were obtained

Phenotypes		Genes		Genotypes		
k+	k—	*K*	*k*	*KK*	*Kk*	*kk*
0 0711	0 9889	0 0362	0 9638	0 0013	0 0698	0 9289

The statistical test shows a close agreement between observed and expected number of families with at least one child of the phenotype k—

The final check of the analysis of k+ × k— matings are given in Table 4 The agreement seems to be satisfactory

Finally, the distribution of families of the mating type k— × k— is presented in Table 5 No exceptions to the theory of inheritance were encountered

TABLE 4

Summary of the K+ × K— Family Material

Test	χ^2	d f	P
N of recessive children given m_e	2 776	1	
Σ me given n	0 156	1	
Total	2 932	2	0 30–0 20

n = total number of families of mating type k+ × K—
m_e = number of families of mating type k+ × K— with at least one K— child

minutes at 37° C, and centrifugation (1000 RPM) was carried out after the addition of Coombs' serum. All bloods, giving positive reactions, were shown to be negative when tested by the direct anti-human globulin technique. The antisera were checked by known positive and negative cells before each series of tests.

RESULTS AND CONCLUSIONS

The results of the investigation are given in Tables 1–5.—The statistical tests were carried out according to the method given by *Smith* (12) for checking the agreement of the observed segregation of a character in *family data with the expected segregation calculated on the basis of* Mendelian ratios and the Hardy-Weinberg formulae.

TABLE 1

Observed Distribution of Families of Mating Type K+ × K—

No. of children in family of group K	No. of children in family tested with anti-K 1	2	3	4	Total
0	1	1	0	1	3
1	0	2	0	0	2
2	0	2	3	0	5
3	0	0	1	0	1
Total no. of families	1	5	4	1	11
Total no. of families with at least one K— child	0	4	4	0	8

TABLE 2

Mating Type K+ × K—
Analysis of Families with at Least One K— Child

No. of children in family	No. of families	No. of K children Obs.	Exp.	Variance
1	0	0	0.000	0.000
2	4	6	5.332	0.888
3	4	9	6.856	1.960
Total	8	15	12.188	2.848

$\chi^2 = 2.812^2 / 2.848 = 2.776$ d.f. = 1 $0.10 > P > 0.05$

In Table 2, *the number of recessive children* is expected to follow a "truncated binomial" distribution. Thus in families of two children from K+ × K— matings, the number of families with 0, 1 and 2 recessives will occur in the ratios 1 : 2 : 1, while in families of three children, the number of families with *0, 1, 2 and 3 recessives* will occur in the ratios 1 : 3 : 3 : 1. However, since families with no recessives have been excluded, the remaining families will occur in the ratios 2 : 1 and 3 : 3 : 1, respectively. It will be seen that in families of three children,

the observed number of K— children was a little higher than expected, resulting in a rather high χ^2 value, but the difference between observed and expected recessives was insignificant

TABLE 3

Mating Type k+ × k—

Analysis of Families with and without Children of Group k—

No of children in family	Total no of families	No of families with at least one k child: Obs	Exp	Variance
1	1	0	0.491	0.250
2	5	4	3.680	0.971
3	4	4	3.436	0.485
4	1	0	0.920	0.073
Total	11	8	8.527	1.779

$\chi^2 = 0.527^2 / 1.779 = 0.156$, d f = 1, 0.70 > P > 0.50

The expected number of families with at least one K— child, recorded in Table 3, was calculated on the basis of the results of a population investigation carried out by *Heiken* (7) This investigation included 4 527 unrelated Swedish children, and the following phenotype, gene and genotype frequencies were obtained

Phenotypes K+	Phenotypes K—	Genes *k*	Genes *k*	Genotypes *kk*	Genotypes *Kk*	Genotypes *kk*
0.0711	0.9889	0.0362	0.9638	0.0013	0.0698	0.9289

The statistical test shows a close agreement between observed and expected number of families with at least one child of the phenotype k—

The final check of the analysis of K+ × k— matings are given in Table 4 The agreement seems to be satisfactory

Finally, the distribution of families of the mating type k— × K— is presented in Table 5 No exceptions to the theory of inheritance were encountered

TABLE 4

Summary of the K+ × k— Family Material

Test	χ^2	d f	P
No of recessive children given m_c	2.776	1	
Σ m_c given n	0.156	1	
Total	2.932	2	0.30-0.20

n — total number of families of mating type k+ × K—
m_c — number of families of mating type k+ × K— with at least one k— child

TABLE 5

Distribution of Families of Mating Type K— × K—

No of children in family tested	Observed no of families	Total no of children	
		K +	K —
1	9	0	9
2	52	0	104
3	24	0	72
4	3	0	12
5	1	0	5
Total	89	0	202

SUMMARY

A material consisting of 100 unrelated Swedish families with 229 children were tested with anti-K in order to study the mode of inheritance of the Kell blood group system The segregation ratios observed were in agreement with those expected, and no exceptions to the genetic theory of the system were encountered

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TRIBUTYRINASE ACTIVITY OF LEPTOSPIRES. FIXED AND SOLUBLE TRIBUTYRINASE DEMONSTRATED BY MEANS OF AN AGAR DIFFUSION TEST

By

HERMAN C ELLINGHAUSEN, JR [1] and OLAV SANDVIK [2]

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Enzymatic studies on leptospires have been hampered by media re- ... pes ... et ... physiological char- acteristics of leptospires which may explain virulence, at least partially so is still warranted Of the leptospiral enzymes reported to date, the lipases have received the greatest attention *Bertok & Kemenes* (1960) reported tributyrinase activity in cultures by pathogens and saprophytes *Parnas et al* (1962) and *Kasarov* (1963) surveyed a number of serotypes for lipase and found that *Leptospira ballum* (*S 102*), *Leptospira autumnalis* (AB Akiyama A), *Leptospira mini* (AB), and *Leptospira sejroe* (*M 84*) were lipase negative *Bertok & Kemenes* (1960) offered the opinion that the lipase activity of certain virulent leptospires might be more valuable than ... ure when ... se activity ... different ... insufficient ... relationship between lipase and virulence of leptospires

A limitation to investigating leptospiral lipase, is the incorporation into media of rabbit serum with its enzyme systems (personal communication, Kemenes 1964) The complicating factor of lipase activity in rabbit serum media has, however, been solved through the recent development of a lipase free medium (*Ellinghausen & McCullough* 1965 b) Taking advantage of these developments, a restudy of leptospiral lipase was initiated

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MATERIALS AND METHODS

Assay of Tributyrinase Activity

A tributyrin Tween 80 agar using nutrient agar as a base was employed for various assays of leptospiral lipase from whole cultures cells sonicated cells culture medium supernatant various whole serums and different species of albumin Tributyrin[1] – 0 086 ml, Tween 80[2] 10 per cent v/v (in distilled H_2O) – 2 15 ml thimerosal 1 per cent w/v (distilled H_2O) – 0 43 ml and distilled water – 5 33 ml were pipetted into a screw cap tube and steamed at 100° C for 5 minutes shaken vigorously for 1 minute while still hot and mixed with 35 ml of nutrient agar[3] at 80 90° C The mixture was poured on a glass plate measuring 12 × 18 cm (agar depth 2 mm) surrounded by plastic strips (0 6 cm thick and 2 cm wide) The plates were leveled prior to pouring of the medium For critical quantitative assays the plates were poured 3 hours before use Circular wells of 5 mm diameter were made in the agar with a cork borer No well was cut nearer to the inner edge of the frame than 2 cm

For some purposes Tween 80 was deleted from the previously described assay medium and its 2 15 ml volume replaced by distilled water In order to achieve emulsification the cold mixture of tributyrin thimerosal and water was emulsified by forcing it through a 27 gauge hypodermic needle 20 times and immediately mixing it with the warm agar and pouring it into plates

From each sample or dilution of sample to be examined a 0 025 ml aliquot was delivered to a corresponding well in the agar plate The agar was covered by another glass plate and incubated in a leveled position for 45 hours at 37° C at which time the diameters of the clearing zones were measured by means of a drafting compass

Comparisons of zones in the same experiment were carried out on the same plate Appropriate geometrical or arithmetrical dilutions were made when doing quantitative assays The highest dilution in a two fold series of dilutions which caused a demonstrable reaction was the tributyrinase titre The quantity of the tributyrinase present in 0 025 ml of this dilution was defined as one diffusion unit of enzyme The diameters of zones were in specific instances used to construct standard curves (*Sandvik* 1962)

Maintenance and Propagation of Leptospires

Cultures were maintained in a medium supplemented with bovine albumin plus 0 20 per cent Aagarose[4] (*Ellinghausen & McCullough* 1965 b) and propagated in the same liquid medium without agar but supplemented with 0 4 per cent dextrose Cultures were incubated at 29° C and aerated by shaking[5] at 140 rpm for usually 7 days Growth was measured as previously described (*Ellinghausen & McCullough* 1965 a)

Dry Weight Determinations and Sonication of Cells

Cultures harvested by centrifugation at 16 500 × G for 30 minutes were washed with phosphate buffer (*Stuart* 1946) The cells suspended in phosphate buffer were
3 at 400 mμ using a Beckmann DU
were dried at 110° C for 24 hours

on) (Optical Density 1 8) were chilled
at n[6] at 1 25 amperes (probe diameter
8 5 ged at
27 000 × G for 30 minutes and the supernatant
original volume All cellular preparations were

1 Eastman Organic Chemicals Rochester New York U S A
2 Atlas Chemicals Wilmington Delaware U S A
3 Baltimore Biological Laboratories Baltimore Maryland U S A
4 Agarose – Bausch & Lomb Inc Rochester New York U S A
5 Shaker produced by New Brunswick Sci Co New Brunswick New Jersey U S A
6 MSE Ultrasonic Power Unit Instrumentation Assoc New York 23, New York U S A

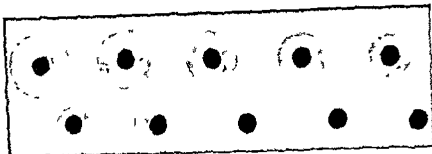


Fig 1
Clearing zones corresponding to a twofold serial dilution of *L pomona* culture supernatant in tributyrin Tween 80 agar medium

Filtration Dialysis and Storage of Culture Supernatants

Ultrafiltration and dialysis (*Ellinghausen & McCullough* 1965 a), were used to concentrate the Tween 80 albumin dextrose medium culture supernatant and to remove diffusible materials from the culture fluids of both strong and weak lipase producers After centrifugation at 16 500 × G for 30 minutes the culture supernatants were for some purposes filtered through serum sterilizing pads (Seitz 100 ma) and Millipore filters (450 and 100 mμ) Supernatants and filtrates were sterility tested observed microscopically for leptospires (subcultured to determine their cell free status) and stored at 5° C and −60° C.

RESULTS

Characteristics of the Assay Reaction

When whole cultures, cell free culture supernatants, or washed cell suspensions of leptospires were placed into wells in the tributyrin agar or tributyrin Tween 80 agar plates, a reaction appeared as a distinct, transparent zone around the well (Fig 1) Clearing in the tributyrin agar could be observed within 10–15 minutes after application of certain samples

With samples low in tributyrinase, the rapidity of clearing was not readily discernible until 24–45 hours of incubation The clearing zones from weak tributyrinase producers were different in that the cleared area on the innermost rim of the well was surrounded by a grayish zone which was more opaque than the unchanged medium However, the cleared zone was distinct and no difficulty was encountered in measuring this clearing The serotypes which brought about this type of clearing zone were *L canicola* (Moulton), *L grippotyphosa* (B 699 Ref 10) *L ballum* (S-102), *L hardjo* (S 91), *L hardjo* (Ref 16) *L grippotyphosa* (Moscow V), *L sejroe* (Mallersdorf), *L naam*, *L mini* (LT 117) *L autumnal*...

Deletion of ...
diffusion con...
phosphate buffer was used to dilute the sample (two fold serial dilution) the zone diameters of clearing decreased gradually with increasing dilution Tween 80 albumin medium (0 1 per cent v v Tween 80)

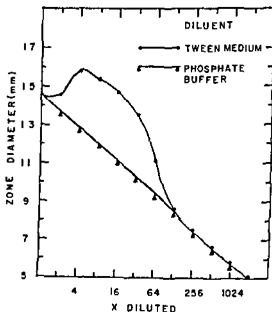


Fig 2

The influence of diluent on the zone diameters of a serial twofold dilution of culture supernatant (*L pomona* Johnson) when tested in a tributyrin agar plate without Tween 80

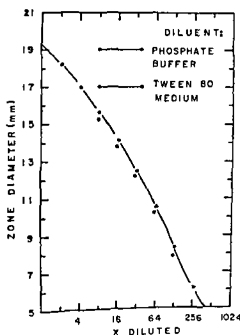


Fig 3

The influence of diluent on the zone diameters of a serial twofold dilution of culture supernatant (*L pomona* Johnson) when tested in a tributyrin Tween 80 agar plate

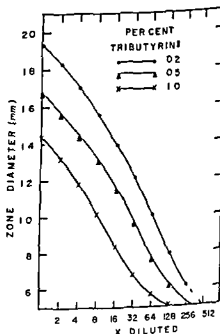


Fig 4

The influence of concentration of tributyrin in the tributyrin Tween 80 medium on the zone diameters of a serial twofold dilution of culture supernatant (*L pomona* Johnson) diluent phosphate buffer

used as a sample diluent, however, tended to bring about an increased clearing zone in the first two dilutions, although the enzyme content was decreased to 25 per cent of the original concentration

When the assay medium was "saturated" with Tween 80 (0 5 per cent v/v) the enhancing effect of the Tween 80-albumin medium as a diluent was practically eliminated (Fig 2 and 3). Although the zone diameters were increased by the presence of Tween 80 (0 5 per cent—Fig 3), the highest sensitivity of the assay was obtained when Tween 80 was omitted from the tributyrin agar (Fig 2) Thus, the end titre for the same enzyme material was higher in the absence of 0 5 per cent Tween 80 in the assay medium The Tween 80 of the culture medium used as a sample diluent did not influence the tributyrinase end titres

In a "saturated' Tween 80 agar medium, varying concentrations of tributyrin in the assay medium had an effect on end titre and zone diameters (Fig 4) The clearing zones with undiluted culture supernatant of *L pomona* (Johnson) were 14 3 and 19 4 mm using 1 per cent and 0 2 per cent tributyrin agar respectively (end titres— 1:128 vs 1 512)

Tributyrinase Titres in Rabbit Serum and Serum Albumins from Various Species of Animals

The tributyrinase titres in rabbit serum and serum albumins from various species of animals are listed (Table 1) Unheated and undiluted rabbit serum had a high tributyrinase titre and even heat inactivation could not completely remove this lipase activity Only rabbit serum albumin contained any tributyrinase activity All of the listed albumins,[1] when employed in a Tween 80 medium, supported the growth of *L pomona* and leptospiral lipase was found in their culture supernatants commensurate with the amount of leptospiral growth

TABLE 1

Tributyrinase Titres in Rabbit Serum and Serum Albumin of Different Species of Animals

Samples	Titres
Rabb[illegible]	
[illegible]	12
[illegible]	28
[illegible]	4
[illegible]	6
[illegible]	6

[1] Albumins purchased from Pentex Incorporated, P O Box 272, Kankakee, Illinois, U S A

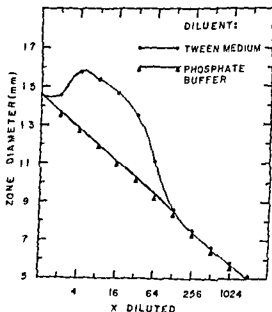


Fig 2

The influence of diluent on the zone diameters of a serial twofold dilution of culture supernatant (*L pomona* Johnson) when tested in a tributyrin agar plate without Tween 80

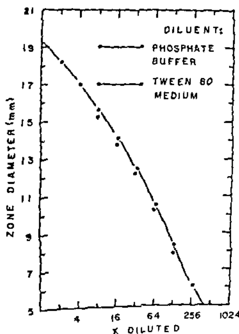


Fig 3

The influence of diluent on the zone diameters of a serial twofold dilution of culture supernatant (*L pomona* Johnson) when tested in a tributyrin Tween 80 agar plate

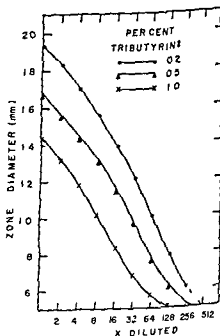


Fig 4

The influence of concentration of tributyrin in the tributyrin Tween 80 medium on the zone diameters of a serial twofold dilution of culture supernatant (*L pomona* Johnson) diluent phosphate buffer

Tributyrinase Production by Various Serotypes of Leptospires

The tributyrinase concentrations of cell suspensions and culture supernatants of 14 leptospiral serotypes are listed (Table 2) All were positive for the cell suspensions Some of the culture supernatants were normally strongly positive for tributyrinase while others had to be concentrated to 1/10 volume in order to result in clearing in the tri-
b [illegible] agar

diffusion unit are noted (Table 2)

The Effect of Cell Washing on the Cell Fixed Tributyrinases

An experiment with *L pomona* (Johnson) summarizes the effect of washing on cellular tributyrinase content (Table 3) The cellular tributyrinase titre remained unchanged after three vigorous washings with phosphate buffer The fluid from the first washing contained only a minute amount of enzyme, although the optical density and the dry weight of a sample of the resuspension was less after washing

TABLE 3

Effect of Washing on the Tributyrinase Content of Leptospira pomona (Johnson) Cells

Sample	Tributyrinase titre	Dry weight			O D 400 m μ (1 10 dilution)
		mg ml	μg 025 ml	μg diffusion unit	
Suspension of unwashed cells	1 128	1 5	37 5	0 294	0 37
Suspension of cells washed 3 times	1 129	1.2	24 0	0 184	0 25
First cell washings	1 16				

Phosphate buffer

The Effect of Cell Disintegration on the Solubilization of Cell Fixed Tributyrinase

Sonication of a heavy suspension of *L pomona* did not reduce the tributyrinase titre of such a suspension (Table 4) Sonication of the starting suspension reduced the optical density and the cell numbers A 1 10 dilution of the initial suspension (1 8 O D) had an optical density of 0 29 and 0 28 with the Beckman DU and Bausch and Lomb Spectronic 20 colorimeter and a nephelometer reading of 55 After sonication direct turbidimetric readings were possible without a 1 10 dilution (0 58 O D and 0 50 O D with the Beckman and Bausch and Lomb and 98 with the nephelometer)

TABLE 2

Tributyrinase Concentrations in Leptospiral Cells and Culture Supernatants

Serotype	Nephelometer reading of 7-day growth	Cell suspensions					Lipase supernatant titre	
		O.D. 400 mμ	mg/ml	μg/0.5 ml	Titre	μg dry weight/diffusion unit	Nonconcentrated	10 x concentrated
L. pyrogenes Salinem	110	1.75	1.59	39.8	1:128	0.312	1:2048	
L. pomona H DMs NADL	110	1.75	1.63	40.7	1:256	0.159	1:512	
L. pomona Johnson	85	1.80	1.65	41.4	1:128	0.322	1:256	
L. australis A Ballico	108	1.74	1.72	42.9	1:128	0.336	1:512	
L. canicola Moulton	125	1.72	1.49	37.2	1:64	0.582	1:256	
L. bataviae Van Tienen	117	1.65	1.68	42.1	1:64	0.659	1:256	
L. grippotyphosa (Ref. 10)	102	1.72	1.59	39.8	1:16	2.49	1:16	
L. ballum S 102	78	1.70	1.42	35.5	1:8	4.45	0	1:2
L. icterohaemorrhagiae AB Wijnberg	80	1.50	1.46	36.7	1:8	4.58	1:16	
L. hardjo LSU 91	60	1.70	1.62	40.5	1:8	5.06	0	1:1
L. hardjo (Ref. 18)	73	1.80	1.71	42.8	1:8	5.35	0	1:1
L. grippotyphosa Moscow V	105	1.65	1.84	46.2	1:8	5.76	1:4	
L. sejroe Mallersdorf	60	1.64	1.46	36.7	1:4	9.18	0	1:1
L. naam (Ref. 1)	30	1.81	1.78	44.6	1:4	11.1	0	
L. mini LT 117	62	1.64	1.71	42.9	1:2	21.4	0	1:1
L. autumnalis AB Akiyama A	85	1.72	1.74	43.6	1:2	21.8	0	1:1
L. hyos	49	1.82	2.05	51.2	1:2	25.6	0	1:1

of the more defined environment in which our cultures were studied. The clearing effect in tributyrin agar was earlier described for staphylococci and other lipolytic bacteria (*Davies* 1954), (*O Leary & Weld* 1964), and for *Mycoplasma* (*Rottem & Razin* 1964)

Diffusion in tributyrin agar demonstrates "preformed" tributyrinase in leptospiral cultures, it being possible to detect the tributyrinase activity brought about by as little as 0.15 µg of leptospiral cellular material. The incorporation of Tween 80 in the assay medium influences the pattern of clearing in the tributyrin agar and, where quantitative comparisons are important, "saturation" of the agar with Tween 80 is mandatory (Figs 2 and 3). When comparing concentrated samples of enzyme, a difference in enzyme content will reflect a considerably higher difference in zone diameters on the Tween "saturated" medium than on a Tween 80 free medium. The tributyrinase titres are probably higher on a Tween 80 free medium because the greater degree of diffusion in the contrasting Tween "saturated" medium tends to spread quickly a minute amount of enzyme over a comparatively wide area.

Decreasing concentrations of tributyrin in the assay medium resulted in increased diameters of clearing zones and, thus, a more sensitive assay system. Practical considerations of visually detecting the clearing process require a concentration of tributyrin of at least 0.2 per cent (v/v)

Our studies of the tributyrinase concentrations in rabbit serum emphasize the probable difficulties of doing lipase studies with leptospiral cultures, even when the whole serum or serum medium is heat inactivated.

Detection of minute amounts of lipase would be difficult in media having intact serum lipase. The use of a Tween 80 albumin medium (*Ellinghausen & McCullough* 1965 b) makes available a medium free of lipase activity in which further work can de done on both the soluble and cell bound lipases of leptospires.

All of the leptospiral serotypes in our study produce tributyrinase in contrast to earlier reports (*Kmety & Bakoss* 1961, *Parnas et al* 1962, and *Kasarov* 1963) wherein *L sejroe, L mini, L autumnalis*, and *L ballum* were non lipolytic. Large quantitative differences in lipase activity exist between various serotypes.

The leptospiral tributyrinase exist in two different states, one "extracellular soluble" and one "cell bound". The extracellular tributyrinase is liberated into the culture supernatant as a function of progressive growth and inoculum size and will be the subject of a future publication. This soluble state is in contrast to the tributyrinase of *Mycoplasma* (*Rottem & Razin* 1964) where the enzyme was not bound to the cell membrane and a[illegible]

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TABLE 4
Effect of Sonication on the Solubilization of Tributyrinase in Leptospira pomona (Johnson)

Sample	Tributyrinase titre	O.D. 400 mμ	Cell count ml
Cell suspensions before sonication	1:128	1.82	6.7 × 10^8
Same suspension after sonication	1:128	0.582	2.4 × 10^4 approximate
First phosphate buffer cell washings	1:32		
Centrifuged cellular debris from sonication of suspension (Restored to 22 ml volume)	1:128		
Supernatant after centrifugation of sonicated cells	1:32	0.078	

The Effect of Filtration, Dialysis, and Storage on Leptospiral Tributyrinase

After centrifugation at 16,500 × G for 30 minutes, leptospiral cells were still present in culture supernatants, but could be removed by a 100 mμ membrane filter and not a 450 mμ filter.[1] Seitz filtration (30 ml filter—100 mμ—3.5 cm pad and 100 ml filter—100 mμ—6.0 cm pad) was of limited value. A 75 per cent reduction in enzyme concentration resulted when small volumes (10.0 ml) of supernatant were passed through the 3.5 cm filter. Large volumes (300 ml) could be filtered with no reduction in enzyme titre but were not cell free.

The tributyrinase in culture supernatant of *L. pomona* (Johnson) was not dialyzable or ultrafilterable. When supernatant was concentrated to 1/10 of its original volume, the end titre increased from 1:64 to 1:512. Culture supernatant stored 9 months at —60° C maintained its tributyrinase end titre. After 3 months at 5° C, there was a 28 per cent reduction in concentration of tributyrinase. Rotary shaking of tributyrinase supernatant at 29° C for 7 days resulted in a 43 per cent reduction. A 10 per cent reduction in enzyme occurred in static controls after 72 hours and then remained constant. When whole cultures of *L. pomona* were shaken for 37 days, enzyme concentration did not change significantly.

No reduction in end titre of supernatant occurred when exposed to temperatures of 30° C to 68° C for 10 minutes at each temperature. All activity was destroyed by heating at 88° C for the same time.

DISCUSSION

Tributyrinolytic activity by leptospires is inactivated by heat, and therefore, we agree with *Bertok & Kemenes* (1960), who refer to it as a "tributyrinase" and we believe it is quite justified in our study because

1 HA type, 0.45 μ, 13 mm. Millipore Filter Corp. Bedford. Mass., U.S.A.

L pyrogenes, *L pomona*, *L australis*, *L canicola*, *L bataviae*, *L grippotyphosa* and *L icterohaemorrhagiae*

5 Deletion and incorporation of Tween 80 in the tributyrin agar medium influenced the diffusion conditions for the tributyrinase

6 A concentration of 0 2 per cent tributyrin in the agar allowed ready detection of clearing

7 The method of assay is sufficiently sensitive to detect the clearing in the tributyrin agar brought about by as little as 0 16 µg of cellular material

8 Through sonication of leptospires it was found that the intracellular tributyrinase is tightly cell bound

9 The tributyrinase of *L pomona* is not dialyzable, very stable when stored at —60° C moderately stable at 5° and 29° C, not diminished in its activity when exposed to temperatures of 30° C to 68° C and destroyed by heating at 88° C (10 minutes)

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Mycoplasma are in contrast to our findings that the major activity of the leptospiral tributyrinase is found in the resuspended cellular debris (27 000 × G 30 minutes) Those leptospiral cultures exhibiting the greatest concentration of cell bound tributyrinase were generally the best producers of soluble enzyme Whether there is a difference between the cellular and extracellular leptospiral lipases has yet to be determined The particle bound state of the cell fixed tributyrinase is however a striking characteristic which was not destroyed by repeated washing of whole leptospiral cells sonication or freezing and thawing Electron microscopic observations of phosphate buffer lysed *L pomona* (*Ritchie & Ellinghausen* 1965) show most of the protoplasmic mass removed and the enveloping sheath of the organism absent These observations would exclude the membraneous sheath enveloping the leptospire as a major source of cell bound tributyrinase When *L pomona* was grown in a synthetic medium (*Stalheim & Wilson* 1964) the cells were found to contain tributyrinase while lipase of a soluble nature was not found in the culture supernatant concentrated ten fold (*Stalheim* 1965 personal communication) In this nonprotein medium it may be that the lipases are liberated into the culture supernatant but are in turn denaturated by the Tween 80 and Tween 60 present *Rottem & Razin* (1964) detected no lipase activity in their mycoplasma growth medium after removal of organisms while with leptospires we have found a high concentration of tributyrinase solubilized into the culture supernatant With the *Mycoplasma* it was felt that the high acidity developed in the growth medium might inactivate the enzyme while this is not generally a problem with leptospires

Dialysis ultrafiltration sonication storage at low temperature and stability at room and incubation temperature of leptospiral tributyrinase suggest that enzymes will lend themselves easily to manipulation without the handicaps encountered with many other enzymes

The quantitative difference in tributyrinase activity among leptospires is quite striking and may be a guide to the further study of leptospiral physiology and their ability to penetrate cells and become intracellular (*Rose et al* 1965)

SUMMARY AND CONCLUSION

1 A diffusion method was developed for the demonstration and titration of leptospiral tributyrinases

2 A bovine albumin Tween 80 growth medium free of lipase activity was an aid in these studies

3 Tributyrinase was demonstrated in the cells of all leptospires including *L pyrogenes L pomona L australis L canicola L bataviae L grippotyphosa L ballum L icterohaemorrhagiae L hardjo L sejroe, L naam L mini L autumnalis* and *L hyos*

4 Extracellular tributyrinase was present only from cultures of

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The Department of Bacteriology (Head Professor Gunnar Lofstrom M D)
University of Uppsala Sweden

IMMUNOFLUORESCENCE IDENTIFICATION OF SALMONELLA IN FAECAL SPECIMENS[1]

By

ABERRA DEMISSIE

Received 10 iv 65

The fluorescent antibody technique was first used in demonstrating soluble pneumococcal polysaccharide in tissue of infected mice in 1942 by *Coons et al* (2)

Since then several workers (4 8 9 10 19 20) have used the method in demonstrating and identifying different organisms from various sources *Whitaker et al* (24) applied the technique to the identification of microorganisms and they studied especially *E coli* 0127 B 8 in four year old samples of faeces collected during an epidemic caused by these bacteria

Thomason et al (23) studying pure culture of *S typhi* were able to demonstrate O Vi and H antigens separately by this technique and subsequently (22) they identified salmonellae in faecal specimens although they encountered serious problem of nonspecificity

The problem of cross reactivity was further evidenced by the recent work of *Silliker et al* (21) when they isolated non salmonellae bacteria which possessed characteristics similar to those of salmonellae

In the present work fluorescein isothiocyanate (FITC) labelled globulins were used for the diagnosis of salmonella bacteria from faeces and river water The results obtained were compared with findings by conventional cultural and serological methods When "false positive results were observed attempts were made to isolate cross reacting bacteria which were thereafter used for absorption

MATERIAL AND METHODS

Reference Strains

Thirty eight salmonellae strains representing all groups through to 0–57 as well as three Arizona strains 89 56 88 56 and 108 56 were kindly provided by SBL (The National Bacteriological Laboratory Stockholm)

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[1] This investigation was made possible thro [illegible]

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was dissolved in distilled water, and dialysed overnight against cold running water in a cold room (4° C)

Fluorescein isothiocyanate (FITC) (Baltimore Biological Laboratory Inc, Baltimore, USA) was conjugated with immune globulin according to the method of

Fluorescent Microscopy Equipment

A Zeiss Fluorescent Microscope with an Osram HBO 200 high pressure mercury-vapour lamp was used A BG (3 mm) ultraviolet input filter was used as a primary filter and a Zeiss 47 or a Zeiss 50 as a secondary filter

The number of fluorescent bacteria was estimated as follows:

Infrequent (I) 1 2 fluorescent bacteria / field of vision
Frequent (F) 3 50 fluorescent bacteria / field of vision
Abundant (A) 50 or more fluorescent bacteria / field of vision

The grading of bacterial fluorescence intensity was determined as:

Peripheral staining of bacteria	3 4+
Solid staining of bacteria	2–3+
Spotted staining of bacteria	1 2+

Only a 3 4 type of staining was considered as a specific antigen antibody reaction

Absorption Techniques

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RESULTS

Antisera

Table 1 shows the results of the tube agglutination tests It is evident that sera produced by immunizing rabbits with heat killed bacteria showed lower titres than those with formalin killed bacteria These latter sera gave high titres with heat killed bacteria containing O-antigens only as well as with formalin killed bacteria possessing both O- and H antigens

The higher titre evidenced under "Formalin killed" might thus be a result of the presence of the H antigens in addition to the O-antigens The significance of this circumstance for the FA-staining is reflected in Figures 1 and 2

Thriteen strains of Brevibacteria were kindly forwarded by Dr *Sillier* of the Department of Pathhology, S James Hospital Chicago, USA

Seven *E coli* strains, 0444, 0125 a, 0119 b and four other coli strains were obtained from stock cultures of this laboratory

Faecal and Water Specimens

Two hundred faecal specimens were received from routine laboratory work of which one hundred were examined by unabsorbed conjugated globulins and one hundred by conjugated globulins after absorption Seventy-one specimens of water were collected from the river Fyris which runs through the town of Uppsala (6)

Preparation of Smears for the FA Technique

Water specimens Varying quantities of washings from swabs submerged in the river Fyris were enriched in Kauffmann's tetrathionate broth overnight at 37° C Smears to be stained were prepared from sediment of the cultures suspended in physiological saline

Faecal specimens About one gram of faecal material was suspended in about 2 ml of physiological saline The emulsified suspension was left in the incubator (37° C) for about two hours after which time it was taken out and smears were prepared for staining

The fluorescent antibody (FA) technique was performed according to the method of *Moody et al* (15, 16) Control smears stained with labelled homologous immune globulins were prepared simultaneously

The titre to be adopted of the immune globulins was determined by titration against the reference strains Controls for serological cross reactions were also established in this way

Strains to be sused as control and reference antigens were grown overnight in ordinary broth The organisms were treated with formalin (1 per cent final concentration) for about two hours at room temperature, after which they were centrifuged at 3 000 r p m for 20 minutes The sediments were suspended in physiological saline and smears were prepared from these suspensions for staining

The same procedures were employed in staining smears of pure cultures of newly isolated strains

Specimens from faeces or river water which were found to be positive by conventional methods but negative by the FA method were enriched in Kauffmann's tetrathionate broth Smears for staining were then prepared from these enriched samples

From specimens which were negative by conventional methods but positive by the FA method it was possible in several cases to isolate strains which were responsible for the "positive" outcome of the test but which did not belong to the salmonellae species In the false positive cases the isolated strains were classified as far as was possible, and used to absorb their respective sera

Isolation of Bacteria by the Conventional Methods

The conventional metheds of isolating salmonellae by culture from river water and faeces has been described in an earlier paper (6)

Preparation of Antisera

Rabbit antisera specific for the O antigens of the Salmonellae groups of B C D and E were kindly forwarded by SBL (The National Bacteriological Laboratory Stockholm)

Antisera were also produced by immunizing rabbits with heat killed or formalin inactivated antigens The method of immunization of the rabbits was that described by *Edwards & Ewing* (7)

Preparation of Fluorescent conjugates

by the use of formalin pared to sera produced era were obtained by fractionation with about 40 per cent saturated ammonium sulfate The precipitate

was dissolved in distilled water, and dialysed overnight against cold running water in a cold room (4° C)

Fluorescein isothiocyanate (FITC) (Baltimore Biological Laboratory Inc, Baltimore, USA) was conjugated with immune globulin according to the method of *Coons & Kaplan* (3) Unconjugated substance was removed by passing the conjugate through a column packed with Sephadex G 25 (AB Pharmacia, Uppsala, Sweden) in phosphate buffered physiological saline at pH 7 2 The conjugated sera were stored frozen in small tubes fitted with rubber stoppers at —20° C.

Fluorescent Microscopy Equipment

A Zeiss Fluorescent Microscope with an Osram HBO 200 high pressure mercury-vapour lamp was used A BG (3 mm) ultraviolet input filter was used as a primary filter and a Zeiss 47 or a Zeiss 50 as a secondary filter

The number of fluorescent bacteria was estimated as follows:

Infrequent (I) 1 2 fluorescent bacteria / field of vision
Frequent (f) 3-50 fluorescent bacteria / field of vision
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The grading of bacterial fluorescence intensity was determined as.

Peripheral staining of bacteria	3-4+
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Spotted staining of bacteria	1-2+

Only a 3 4+ type of staining was considered as a specific antigen antibody reaction

Absorption Techniques

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RESULTS

Antisera

Table I shows the results of the tube agglutination tests It is evident that sera produced by immunizing rabbits with heat killed bacteria showed lower titres than those with formalin killed bacteria These latter sera gave high titres with heat killed bacteria containing O-antigens only as well as with formalin killed bacteria possessing both O- and H-antigens

The higher titre evidenced under "Formalin killed" might thus be a result of the presence of the H antigens in addition to the O antigens The significance of this circumstance for the FA-staining is reflected in Figures 1 and 2

TABLE 1

Tube Agglutination Comparison between Sera Produced by Immunizing Rabbits with Formalin and Heat Killed Antigens

Anti salmonella group sera	Treatment of antigen	Strains used for agglutination	Agglutination with heat killed bacteria	Agglutination with formalin killed bacteria
B	Heat killed	*S. paratyphi B*	1:128	
C		*S. newport*	1:256	
		S. thompson var. berlin	1:128	
D		*S. typhi*	1:128	
		S. enteritidis	1:128	
E		*S. london*	1:32	
		S. newington	1:128	
B	Formalin killed	*S. paratyphi B*	1:1024	1:1024
C		*S. newport*	1:1024	1:2048
		S. thompson var. berlin	1:2048	1:4096
D		*S. typhi*	1:2048	1:4096
		S. enteritidis	1:2024	1:2048
E		*S. london*	1:1024	1:2048
		S. newington	1:2048	1:4096

S. Newport (6,8: eh: 1,2) was stained with anti salmonella serum C. The reaction was graded as 3-4+, mainly because of the distinct staining of the wall regions of the bacteria. The reaction was estimated as negative when *S. Chingola* (11: eh: 1,2) was stained with the same serum C. This salmonella type possessed flagellar antigens but no O antigen in common with *S. Newport*. Flagellar staining was observed as an extracellular, hazy background illumination and to some extent as distinct stained flagella (Fig. 2).

Evaluation of the fluorescent antibody technique on pure cultures

Salmonella

Table 2 shows the results of the fluorescent antibody technique in comparison with the slide agglutination procedures using unabsorbed sera. The same result after absorption is recorded in Table 3.

In general there were no serious cross reactions. The only one which was noticed occurred when a strain of the B group was stained with labelled immune globulins of D group as these groups share common O antigens (antigens O1 and O12).

No fluorescence was induced by non immune rabbit globulin conjugate.

Evaluation of Labelled Anti Salmonella O Sera in the Diagnosis of Salmonella Bacteria in River Water

Seventy one specimens of river water were tested by the FA method and the results are presented in Table 4. In the groups B, C and D



Figs 1 2

Fig 1 Pure culture of S newport (c) (6 8 e h 1 2) treated with FITC con ugated action

Fig 2 ugated Tri X

TABLE 1

Tube Agglutination Comparison between Sera Produced by Immunizing Rabbits with Formalin and Heat Killed Antigens

Anti salmonella group sera	Treatment of antigen	Strains used for agglutination	Agglutination with heat killed bacteria	Agglutination with formalin killed bacteria
B	Heat killed	*S paratyphi B*	1 128	
C	„	*S newport*	1 256	
	„	*S thompson var berlin*	1 128	
D	„	*S typhi*	1 128	
	„	*S entritidis*	1 128	
E	„	*S london*	1 32	
		S newington	1 128	
B	Formalin killed	*S paratyphi B*	1 1024	1 1024
C	„	*S newport*	1 1024	1 2048
	„	*S thompson var berlin*	1 2048	1 4096
D	„	*S typhi*	1 2048	1 4096
	„	*S entritidis*	1 2024	1 2048
E	„	*S london*	1 1024	1 2048
	„	*S newington*	1 2048	1 4096

S Newport (6,8 eh, 1,2) was stained with anti-salmonella serum C The reaction was graded as 3–4+, mainly because of the distinct staining of the wall regions of the bacteria The reaction was estimated as negative when *S Chingola* (11 eh 1,2) was stained with the same serum C This salmonella type possessed flagellar antigens but no O antigen in common with *S Newport* Flagellar staining was observed as an extracellular, hazy background illumination and to some extent as distinct stained flagella (Fig 2)

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No fluorescence was induced by non-immune rabbit globulin conjugate

Evaluation of Labelled Anti-Salmonella O-Sera in the Diagnosis of Salmonella Bacteria in River Water

Seventy-one specimens of river water were tested by the FA method and the results are presented in *Table 4* In the groups B, C and D

TABLE 3

Slide Agglutination and Staining by Conjugated Antisalmonella Rabbit Sera B, C, D and E and Normal Rabbit Serum of Serotypes of Salmonella and the Relevant Arizona 89/56 after Absorption with Crossreacting Strains (see Table 2)

Strains	Conjugated antisalmonella sera								Normal rabbit	
	B		C		D		E			
	FAT	ACG	FAT	ACG	FAT	ACG	FAT	ACG	FAT	ACG
S. paratyphi A	1-2+	–	–	–	–	–	–	–	–	–
S. typhimurium	3-4+	+++	–	–	–	–	–	–	–	–
S. newport		–	3-4+	+++	–	–	–	–	–	–
S. enteritidis	–		–	–	3-4+	+++	–	–	–	–
S. london	–		–	–	–	–	1-4+	+++	–	

A comparison between the fluorescent antibody technique and slide agglutination in the diagnosis of various salmonella types after absorption with crossreacting strains (see Table 2)

TABLE 2

Slide Agglutination and Staining by Conjugated Antisalmonella Rabbit Sera and normal Rabbit Serum of Various Specimens of Gramnegative Bacteria and the Relevant Serotypes of Salmonella before Absorption

Strains	Conjugated antisalmonella sera								Normal rabbit serum	
	B		C		D		E			
	FAT	AGG	FAT	AGG	FAT	AGG	FAT	AGG	FAT	AGG
S. paratyphi A	1 2	–		–	–	++	–	–	–	–
S. typhimurium	3 4	+++		–	3–4*	+++	–	–	–	–
S. newport		++	3–4	+++	–	++	–	–	–	–
S. enteritidis			–	–	3–4*	+++	–	–	–	–
S. london			–	–	–	–	3 4*	+++	–	–
Arizona 88/56				–	–	–	–	–	–	–
Arizona 89/56	1 2				–	–	–	–	–	–
Arizona 108/56					–	–	–	–	–	–
E. coli 044			–			–	–	–	–	–
E. coli 0125				–	–	–	–	–	–	–
E. coli 0119				–	–	–	–	–	–	–
Proteus (2)			–	–	1*	–	–	–	–	–
Aerobacter (2)				–		–	–	–	–	–
E. coli (4)	–					–	–	–	–	–

FAT = fluorescent antibody technique
AGG = agglutination
A study on crossreactions in unabsorbed sera for the *Salmonella* groups B, C, D and Γ on the relevant *Salmonella* strains and some other gramnegative bacteria by the use of fluorescent antibody technique *and* slide agglutination

Almost every faecal specimen contained bacteria which gave positive sult with unabsorbed FA globulins, and on several occasions the icteria were positive for more than one globulin Of the hundred iecimens examined, a total of one hundred and ten smears were found i be positive It is seen from Table 5 that twenty three samples were ound to be positive for the B group by the FA method compared with our isolations by the conventional method For the C group the results ere fourty four by the FA and three by the conventional method, for he D group fourty three by the FA and fourteen by the conventional nethod and for the E group twenty two by the FA and zero by the onventional method

The isolated salmonella strains were two *S typhimurium*, one *S tanley* and one *S chester* belonging to the B group, two *S newport* and one S *manchester* belonging to the C group, and ten *S entritidis* and four S *typhi* belonging to the D group It was however, evident that unabsorbed sera caused a large number of false positive results

Typing of Cross Reacting Strains

Some fifteen different strains of bacteria were isolated which caused false positive results Nine of these strains were *E coli* and through the courtesy of Dr *Frits Ørskov*, the International Escherichia coli Center, the National Serum Institute Copenhagen, Denmark, they were typed and the result is summarized in Table 6

One strain was diagnosed as *Klebsiella*, two as *E coli freundii*, one as *Proteus mirabilis*, one as *Bethesda Ballerup* and one as *Pseudomonas aeroginosa* The *Pseudomonas* was isolated from river water, as mentioned before and the fourteen others from faecal specimens Table 7

TABLE 6

Typing of Crossreacting Strains Isolated from Faecal Specimens §

Strain	Name of strain biochemical	Sera
1	*F coli*	09 K(A) H –
2	"	018 K? H 14
3		Spontaneous agglutination H 10 } biochemically identical
4	"	Spontaneous agglutination H 10 } biochemically identical
5	"	0112 (0123) K? * neg in H 1 H 49
6	"	(083) K? H this strain showed weak reaction in O sera 022 093 and 095
7	"	O147 K? H 28
8	"	Weak reaction in 04 018 0147 K? H 30
9	"	075 K? H culture spontaneous agglutination

* [illegible] 1 → H 45 sera

§ [illegible] Dr F Ørskov

twenty-six specimens were positive by the FA method and nine with the conventional method, which roughly gives a yield approximately three times higher for the former. For the E group eleven samples were positive by the fluorescent antibody method against none by the conventional methods. An attempt was made to isolate the organisms responsible for the positive results.

TABLE 4

Fluorescence of Smears Prepared from Swab Washings Enriched in Kauffmann's Tetrathionate Broth

Method	No. of specimens	Conjugated antisalmonella sera								Total positives specimens		Normal rabbit serum	
		B		C		D		E					
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
FA	71	9	12.7	14	19.7	3	4.2	11	15.5	37	52.1		–
C	71	3	4.2	5	7.1	1	1.4	–	–	9	12.7		–

FA = Fluorescence Antibody
C = Conventional

When smears, prepared from individual colonies on Endos plates, were stained, it was possible to isolate a strain of *Pseudomonas aeroginosa* which was brilliantly stained by anti-salmonella serum E. Further investigation revealed that this strain possessed an 0–10 antigen, which is also found among salmonellae of the E group.

Labelled anti-salmonella E was then absorbed by *Ps. aeroginosa*, and when the original eleven smears were again stained, nine (90.0 per cent) became negative. The sera for B, C and D were also absorbed with this Pseudomonas strain, but the absorption did not affect the results obtained for these groups. No additional gram-negative strains could be isolated which were responsible for false positive reactions in these groups.

Evaluation of Labelled Anti-Salmonella O-Sera in the Diagnosis of Salmonella Bacteria in Faecal Specimens

The results of the findings by FA method compared with the conventional method are presented in Table 5.

TABLE 5

Positive Results of Smears of Faecal Specimens Tested by Conjugated Antisalmonella Sera before Absorption

Method	Specimens tested	Conjugated antisalmonella sera								Total positives		Normal rabbit
		B		C		D		E				
		No.	%	No.	%	No.	%	No.	%	No.	%	
FA	100	23	23	44	44	43	43	22	22	110	110	
C	100	4	4	3	3	14	14	–		21	21	

TABLE 8

FITC Reaction of Strains Isolated from Faecal and Water Specimens to Different Conjugated Anti Enteropathogenic E. coli Sera

Strain	Name of strain (biochemical)	026b	055	0128 B12c	086B	0119b	0125a	0126a	0127	0114b	0111	0112ab	0112ac
1	*E. coli* 09		1			1 2+	1+	1+	1+	–	–	–	–
2	*E. coli* 018	1	1 2		1 2		–	–	–	–	–	–	–
3	*E. coli*			–	–	1 2+	1+	1 2+	1 2+	–		–	–
4	*E. coli*		3 4		–		–		–	–	–	–	–
5	*E. coli* 0112		1 2	1 2			1 2+		1+	1 2+	1+	1+	3 4+
6	*E. coli* (093)	2 3+	3 4			–	–	–	–	–	–	–	–
7	*E. coli* 0147		–			–	–		–	–	–	–	–
8	*E. coli*					–	–	–	–	–	–	–	–
9	*E. coli* 075				–	–	–		–	–	–		–
10	*Klebsiella*	–	–	–						–	3 4+		3 4+
11	*C. freundii*	1+ spotted staining	1+	1	1+	1+	1+	1+	1+	1+	1	1+	3 4+
12	*C. freundii*						–		–		–	–	–
13	*P. mirabilis*	1+	1+	1+	2 3+	1+	1	1+	1	1+	1+	1+	1+
14	*Bethesda Ballerup*	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+
15	*Ps. aeruginosa*	–		–	–	–	–	–	–	–	–	–	–

TABLE 7

Result of FA Staining and Slide Agglutination of Crossreacting Bacteria

Strain	Strain name	FA reaction B	FA reaction C	FA reaction D	FA reaction I	Slide agglutination B	Slide agglutination C	Slide agglutination D	Slide agglutination I	FN
From faeces										
1	*E Coli* 09			–	3-4	ND	ND	ND	–	–
2	*E Coli* 018	3 4+*	3 4+	3-4+	3-4+	–	–	+++	–	–
3	*E Coli*	1	1	2-3+	3-4+	ND	ND	ND	–	–
4	*E Coli*	1	1+	3-4+	1 2+	ND	ND	–	ND	–
5	*E Coli* 0112	3 4	1 2	3 4+	1 2+	ND	ND	+++	ND	–
6	*E Coli* (083)			2 3 4+	–	ND	ND	–	ND	–
7	*E Coli* 0147		–	3 4+	–	ND	ND		ND	–
8	*E Coli*	2 3+	1 2	3 4+	1 2+	ND	ND	–	ND	–
9	*E Coli* 075			3 4+	–	ND	ND	–	ND	–
10	*Klebsiella*			3 4+	–	ND	ND	+++	ND	–
11	*C freundii*			–	3-4+	ND	ND	ND	+++	–
12	*C freundii*		3 4	–	–	ND	–	ND	ND	–
13	*P mirabilis*	3 4	3-4+	3-4+	1 2+	+++	+++	+++	ND	–
14	*Bethesda Ballerup*		3 4	–	–	ND	+++	ND	ND	–
From water										
15	*Ps aeruginosa*			–	3-4+	ND	ND	ND	–	–

CN = Conjugated normal rabbit
ND = Not done
* Italic figures denote the strains which after pooling have been used for absorption

2 4 5, 6, 7, 8, 9, 10 and 13 for D serum and strains 1, 2, 3, 11, 15 for the E serum, according to procedures described above

Typing of faecal Specimens with Absorbed Conjugated Anti Salmonella Globulins

After absorption the results of the FA technique and the conventional methods closely agree for groups B, C and D (Table 10) For the E group, however, six specimens were found to be positive by the FA technique while no salmonellae of this group were isolated by the conventional method

TABLE 10

Positive Results of Smears of Faecal Specimens Tested by Conjugated Antisalmonella Sera after First Absorption

Method	Specimens tested	Absorbed conjugated antisalmonella sera								Total positives		Normal rabbit
		B		C		D		E				
		No	%	No	%	No	%	No	%	No	%	
FA	100	28	28	8	8	6	6	6	6	48	48	–
C	100	26	26	5	5	5	5	–	–	36	36	–

TABLE 11

Classification of Salmonellae Strains Isolated from Faecal Specimens

Salmonella groups	Classification of strains		No of specimens	No of patients
B	*S typhi murium*	(11)	11	6
	S haifa	(2)	2	1
	S abony	(1)	1	1
	S stanley	(3)	3	1
	S chester	(1)	1	1
	S brandenburg	(4)	4	1
	S reading	(4)	4	1
C	*S newport*	(3)	3	1
	S manchester	(2)	2	1
D	*S enteritidis*	(8)	8	1
	S typhi	(4)	4	1

Table 11 gives the result of the isolation by culture of the various thirty six salmonella strains The results for the B, C and D groups were considered to justify a more extensive trial as to the final specificity of the FA technique Attempts were made without success, to isolate the bacteria which were responsible for the positive reactions within group E ...

presen

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summarizes the results of FA staining of the actual strains supplemented with slide agglutination. As seen in the table, three strains (two *E. coli* and one *P. mirabilis*) gave 3–4+ reactions with conjugated anti-salmonella B globulins. The results for the C group were four strains (one *E. coli*, one *C. freundii*, one *P. mirabilis* and one *Bethesda-Ballerup*), for the D group nine strains (seven *E. coli*, one *Klebsiella*, one *Proteus mirabilis*) and for E group five strains (three *E. coli*, one *C. freundii* and one *Ps. aeroginosa*). The results of the slide agglutination did not quite agree with that of the FA method. Strains one, three, four, nine and twelve, which were brilliantly stained by the FA method, failed to agglutinate. Strains two, six, seven and eight also failed to agglutinate but the FA staining was somewhat atypical for these strains.

An attempt to type the fifteen cross-reacting strains by the FA technique was made using some available conjugated anti-*E. coli* sera. Three of the nine *E. coli* were typable (Table 8). Strains nos. 4 and 6 were found to react with group 0 55 antiglobulin and No. 5 was positive with group 0–112ac. The difference in result between the FA test and the tube agglutination test can not be explained and calls for further studies.

TABLE 9

Tube Agglutination of Strains Found to be Positive by the FA Method with Conjugated Anti-Enteropathogenic E. coli Sera

No. of strain	Name of strain	Conjugated anti entropathogenic *E. coli* sera		
		055	0111	0112ac
4	*E. coli*	1:16	ND	ND
5	*E. coli*	ND	ND	1:64
6	*E. coli*	1:32	ND	ND
10	*Klebsiella*	ND	1:8	1:8
11	*C. freundii*	ND	ND	1:8

ND = Not done

Strain no. 10, biochemically identified as *Klebsiella*, was stained with conjugated anti-*E. coli* 0 111 and 0 112ac sera. This outcome may have been due to the fact that *Klebsiella* share a common antigen with *E. coli*. The same may be said of strain no. 11 (*C. freundii*), which showed a spotted staining with all the sera, and was brilliantly stained by anti-*E. coli* serum 0 112ac. It should also be noticed that the above results are confirmed by the results obtained with tube agglutination found in Table 9.

Absorption

The different strains which were found to be responsible for the "false positive" results were used for absorption. Thus, strain 2, 5 and 13 (Table 7) were used to absorb the conjugated B serum, strains 2, 12 and 14 were used for absorption of anti-salmonella C serum, strains

2, 4, 5, 6, 7, 8, 9, 10 and 13 for D serum and strains 1, 2, 3, 11, 15 for the E serum, according to procedures described above

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		B		C		D		E				
		No	%	No	%	No	%	No	%	No	%	
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	S brandenburg	(4)	4	1
	S reading	(4)	4	1
C	*S newport*	(3)	3	1
	S manchester	(2)	2	1
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	S typhi	(4)	4	1

Table 11 gives the result of the isolation by culture of the various thirty-six salmonella strains The results for the B, C and D groups were considered to justify a more extensive trial as to the final specificity of the FA technique Attempts were made, without success, to isolate the bacteria which were responsible for the positive reactions within group E Salmonellas of this group are rare in Sweden, and the results presented here might be false positive, due to other cross-reacting bacterial strains, which so far have not been isolated

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6	*E coli*	1 32	ND	ND
10	*Klebsiella*	ND	1 8	1 8
11	*C freundii*	ND	ND	1 8

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DISCUSSION

The purpose of the present study was to explore the potential usefulness of the fluorescent antibody method for the rapid screening of salmonellae in smears prepared from faecal specimens and river water by the use of conjugated anti-salmonella O-globulins A system must be sensitive and specific if it to be used successfully In this study no case which was positive by the conventional method was missed by the screening with thee FA technique There is evidence that the latter was somewhat more sensitive, which is seen from the results with the groups B, C and D after absorption of the globulins

The lack of *serological specificity* was *tackled by isolating* the crossreacting bacteria and by absorbing the globulins It is possible to isolate probably all strains which in this material cause cross-reaction with the groups B, C and D The absorption of the respective sera resulted in highly specific factor sera For group E at least one cross reacting strain was not found, and the attempt to produce a group specific E serum still remains to be done This question must be studies *in more detail*

It is interesting to note that some of the serologically cross-reacting strains listed above were isolated from patients with diarrhoeae but from whom no salmonella or other of the known causative organisms could be isolated by the conventional method Some of these strains were the colitypes which cause diarrhoeae in infants There is, however, no evidence of etiological relationship between these strains and the current disease

It was observed that higher antisera titres were produced by using formalin instead of heat killed bacteria In spite of the possibility that antisera for the former type might contain antibodies also for H antigens this does not seem to interfere with the results of the FA method Studies are in progress to examine other types of antigen products for the obtained by extracting the bacteria with alcohol and acetone There is some evidence *that antisera produced with these antigens have* high titres and a high degree of specificity These results will be presented in a further paper

SUMMARY

An investigation into the usefulness of the fluorescent antibody *method in* detecting salmonellae in faeces and river water was undertaken Cross serological reactivity was found to be a serious handicap, as evidenced by studying various isolates from faecal specimens *All the* isolated strains belonged to the family Enterobacteriaceae Absorption techniques were employed to minimize the risk of cross-reactivity and the results indicate that the solution of the problem is to be found here

Sera of satisfactory specificity were produced for the Salmonellae

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Acta path et microbiol scandinav 65 287–294 1965

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STUDIES OF SALMONELLA ANTIGENS BY THE AGAR GEL PRECIPITIN TEST

By

T HOLME and L EDEBO

Received 10 iv 65

The analysis of antigens of *Salmonellae* by immunodiffusion methods offers a very complex picture *Whiteside & Baker* (1962) studied antigens in extracts of Salmonella bacteria belonging to the groups A, B, C_3, D_1 and D_2 by means of the agar gel precipitin test In addition to the O, H, and Vi antigens, which were represented by specific precipitation lines, they observed several cross reacting antigens The D group of the Kauffmann White scheme was most extensively studied and the six lines observed in tests using extracts from these cells could all be reproducibly identified One line represented the group specific antigen, containing the determinants 9 and 12 of the Kauffmann-White scheme A second line represented the Vi antigen and a third the flagellar (d) antigen The three remaining antigens were found in several of the strains tested even in those not belonging to the D group Furthermore, one of these antigens was also found in extracts from *Shigella flexneri*, *Shigella sonnei*, *Proteus vulgaris* and *Klebsiella pneumoniae* It was pointed out that the cross-reacting antigens, which apparently do not take part in the usual agglutination reaction, nevertheless are present on the surface of the bacterial cell, since whole organisms can absorb the [illegible]

[illegible]

19[illegible] [illegible] 1962) Degradation of the endotoxin by mild acid hydrolysis resulted in [illegible] specificity (*Ribi et al* [illegible] the different determin[illegible] present in the same molecule

The studies reported in this paper mainly confirm the results obtained by *Whiteside & Baker* (1962) However, in addition to a description of some methodological observations, the pronounced variability in

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Our thanks are due to Mrs Kerstin *Magnusson* and Miss *Berit Lindholm* for their skilled technical assistance This work was aided by a grant from the *Swedish State Medical Research Council*

the antibody responses of the rabbit to antigens of gram negative bacteria is strongly indicated

MATERIALS AND METHODS

Strains The following strains were obtained from the National Bacteriological Laboratory, Stockholm *Salmonella paratyphi A* var *durazzo* (IS 2) *Salmonella paratyphi* BO (non motile IS 248) *Salmonella typhimurium* 395M *Salmonella bonariensis* (IS 145) and *Salmonella typhi* (IS 58) The strain of *Salmonella typhimurium* designated 395M contains the O factors 4 5 and 12 A stable R mutant was isolated from an aged broth culture of this strain A strain of *Salmonella enteritidis* designated S 795 was obtained from Dr K C Milner, Rocky Mountain Laboratory Hamilton Montana, USA The antigenic composition of the strains is given in Table 1

TABLE 1

Antigenic Composition of Strains Used

Salmonella paratyphi A var *durazzo*	(I S 2)	2 12 a —
Salmonella BO (non motile)	(I S 248)	4 5 12
Salmonella typhimurium 395M		4 5 12 i 1 2
Salmonella typhimurium 395M/R		– i 1 2
Salmonella bonariensis	(I S 145)	6 8 i e n x
Salmonella typhi	(I S 58)	9 12 Vi d –
Salmonella enteritidis S 795		1 9 12 g m

End sugar of Antigenic Determinant of O Antigen (Staub et al 1959)

1	glucose	6	?
2	paratose	8	abequose
4	abequose	9	tyvelose
5	acetyl galactose	12	glucose

Immunological techniques Gel precipitin tests were performed according to the techniques described by *Feinberg* (1958 1964) Plates were incubated at 37° C for four days since this was found to be the optimum time Difco agar dissolved in 0 9 per cent sodium chloride was used The agar concentration was one per cent Agglutination tests were performed by serial dilution of sera from the bleedings and adding a bacterial suspension killed by heating at 100° C for 10 minutes as the antigen Titres were read after overnight incubation at 37° C

EXPERIMENTAL

Preparation of antigens for precipitin tests Cell were grown in Erlenmeyer flasks on a rotary shaker at 37° C for 18 hours The medium used was a nutrient broth containing 1 per cent Difco tryptone and 0 5 per cent Difco yeast extract The bacteria were collected by centrifugation and suspended in 0 02 M phosphate buffer to a density of approximately 20 mg per ml (dry weight) They were killed by UV irradiation 0 5 per cent formaldehyde or by heat treatment at 70° C for 30 minutes Cells killed by UV were then treated for 5 minutes and cells killed by the other methods for 15 minutes in a Raytheon 10 kC sonic oscillator The sonicate was centrifuged at 10,000 g for 5 minutes and the supernatant used in the gel precipitin tests Disintegration by freeze pressing (*Edebo* 1961) was also tested, but no difference in the gel precipitin tests was observed with this method as compared to sonic treated cells

For the comparison of different methods for the preparation of an

igens dry weight determinations were performed on dialysed supernatants from the disintegrated cells The method used for killing the cells had a profound influence on the final antigen preparation Formaldehyde-killed cells were highly resistant to disintegration and the yield of high molecular weight substances in the supernatant after sonic treatment was low After heat treatment at 70° C for 30 minutes, as much as 40 per cent of the cell mass was released by sonication as soluble, high molecular weight substances, although at least 90 per cent of the cells were recognizable as rods giving a fairly good contrast under the phase contrast microscope Cells killed by ultra violet irradiation were highly sensitive to disintegration, approximately 75 per cent of the cell mass being released as soluble material by sonic treatment In this case the cells lysed and most of the insoluble materials, sedimented by centrifugation, consisted of cell walls

The gel precipitin tests gave very different results with the different antigen preparations With an amount of 0 5 mg dry weight per well (concentration 5 mg per ml) the heat-treated preparations gave a very distinct line near the well, representing the group-specific antigen Two or three additional lines were also regularly observed with these preparations representing heat-stable antigens which were probably not proteins because they showed resistance to degradation by treatment with alkali

With the UV-killed preparations the line representing the group-specific antigen was very broad and diffuse, and a large number of lines was observed representing heat-stable, as well as heat-labile, an- [illegible]

[illegible]

Preparation of antisera Antisera to *Salmonella typhimurium* and *Salmonella enteritidis* were prepared in rabbits Different antigen preparations of the *S typhimurium* strain were tested for their ability to provoke precipitins demonstrable in the gel precipitin tests In one experiment a formaldehyde killed vaccine prepared from the R mutant was compared with a vaccine prepared from the S form Four rabbits were used for each vaccine No precipitating antibodies could be demonstrated in the sera from rabbits immunized with the R vaccine while the four rabbits injected with the S vaccine all gave precipitation lines in the gel precipitin test with both *R-antigen and S-antigen*

The following types of antigen preparations from the S form were tested whole cells killed by heat, formaldehyde or UV, acetone dried cells and cell wall preparations from UV-killed cells For the preparation of cell walls, cells were suspended in a 0 02 M phosphate buffer to a concentration of 10 mg dry weight per ml and subjected to sonic treatment for 3 minutes in a *Raytheon 10 kC* sonic oscillator at maximum effect It was found necessary to use cells killed by UV and not by heat or formaldehyde for the preparation of cell walls, since the

heat- or formaldehyde-treated cells did not release their cytoplasmic materials after sonic treatment The sonicated suspension was diluted in 5 volumes of distilled water and centrifuged at low speed (1000 g) for 5 minutes The pellet was resuspended and sonicated again, diluted and centrifuged The supernatants from the two treatments were combined and washed twice in a 0 02 M phosphate buffer by centrifugation at 10,000 g The resulting suspension consisted of a very homogeneous cell wall suspension, with less than 1 per cent whole cells, which was checked using phase contrast microscopy

The different antigen preparations were tested on groups of three rabbits, three groups usually being run in parallel In these tests a total number of sixty rabbits was used It was found that the cell wall antigen was superior to other antigens tested for the preparation of antisera for gel precipitin tests, both in the percentage of rabbits giving a positive response and in the number of precipitation lines obtained with the sera The individual response to any antigen was variable, however, and in one out of six rabbits immunized with a formaldehyde killed whole cell antigen, a precipitating serum of equal quality to that using the cell wall antigen was obtained No rabbit immunized with cell wall antigen failed to yield a good precipitating serum, although the pattern in the gel precipitin test showed a high degree of variation *Heat-killed and acetone-dried vaccines gave very poor precipitating* antisera

An additional number of 20 rabbits was then immunized with a cell wall preparation The antisera from these immunizations were kept separate and some of them used as indicators for different antigens which was made possible by the different responses of the individual rabbits

Ten rabbits were also immunized with vaccines prepared from *Salmonella enteritidis,* seven with a cell wall preparation and three with formaldehyde killed whole cells

For immunization, doses of 0 2 mg dry weight were given intravenously twice a week to rabbits with an initial weight of approximately 2 5 kg It was found that the maximum O-agglutinin level was obtained after an immunization period of 4–5 weeks Antisera, giving a maximum number of lines in the gel precipitin tests were obtained after a minimum of 9 weeks No correlation was found to exist between the agglutinin titre and the ability to give precipitation in the gel precipitin test Similar findings have been reported by *Sulitzeanu (1958)* When good precipitation patterns were obtained with sera from test bleedings the animals were exsanguinated The globulins precipitating at half saturation with ammonium sulphate in the cold were then prepared from each antiserum The globulin precipitate was washed once in half-saturated ammonium sulphate and then dissolved in distilled water to approximately one tenth of the original serum volume

Gel precipitin tests An experiment, in which a cell wall preparation

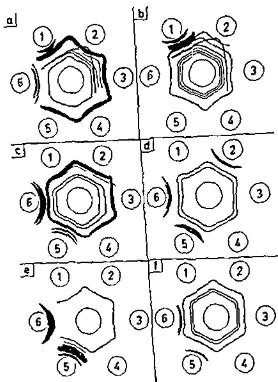


Fig 1

Patterns obtained in the gel precipitation test with antisera from different rabbits

from *Salmonella enteritidis* was used as immunizing agent illustrates the varying response in different rabbits to the different antigenic components of one preparation Seven rabbits were immunized with the cell wall preparation and three rabbits were immunized with formaldehyde killed whole cells of the same organism For comparison two additional rabbits were simultaneously injected with a cell wall preparation from *Salmonella typhimurium* The results from this experiment are shown in Fig 1 Specific responses are represented by the precipitation lines near the antigen wells in homologous systems In addition, the presence of several non specific lines indicate the existence of common antigens in bacteria belonging to different serological groups The three rabbits immunized with formaldehyde treated whole cells showed a different response In addition to a weak line representing the group



Fig 2

Demonstration of cross reacting somatic antigens from Salmonella strains belonging to the serological groups B and C. Precipitating antigens were prepared by heat treatment of cells at 60 C for 30 minutes and sonicated for 15 minutes

A
1 antigen *Salmonella typhimurium* 395M (B group)
2 antigen *Salmonella bonariensis* (C_2 group)
3 antiserum c w 395M
4 antiserum c w 395M absorbed with *Salmonella bonariensis* antigen

B
1 antigen *Salmonella typhimurium* 395M/R
2 antigen *Salmonella bonariensis*
3 antiserum c w 395M
4 antiserum c w 395M absorbed with *Salmonella bonariensis* antigen

specific antigen, there was a strong precipitation line with the *Salmonella enteritidis* antigen showing no reaction of identity with any line with the *Salmonella typhi* antigen. This line could be shown to represent the flagellar antigen, by identity reaction and specific absorption with a flagellar preparation from *Salmonella budapest*[3] (1,4,12 g,t:–)

Another experiment, illustrating the common antigens of Salmonella bacteria belonging to different serological groups is shown in Fig. 2. An antiserum against a cell wall preparation from *Salmonella typhimurium* 395M was used. In the homologous system, three well defined lines developed. The antigens could be heated to 100° C for 30 minutes and treated with 0.02 M NaOH without destruction. Two lines showed reaction of identity with lines from *Salmonella bonariensis*, belonging to group C_2. Absorption with *Salmonella bonariensis* removed these lines but did not interfere with the formation of one line, which therefore was considered as group-specific. The C_2 somatic antigen contains, like the B somatic antigen, abequose, but this similarity was not the cause of the cross reaction. An R mutant of *Salmonella typhimurium* 395M, which showed no presence of abequose when analysed by thin film chromatography using a phenol-water extract, expressed the non-specific lines very clearly. The non motile Salmonella strain, belonging to the B group gave rise to the same precipitation pattern as *Salmonella typhimurium* 395M. This shows conclusively, that the lines do not depend on flagellar antigens.

DISCUSSION

The existence of cross-reacting antigens between different species of enteric bacteria are only to a limited degree revealed by the agglutination reaction. *Lüderitz et al.* (1964) have shown, that the R mutants of different Salmonella bacteria display very few specificities. *Kunin* (1963) has also reported on a common antigen in Enterobacteriaceae. These findings are in good agreement with the similarity in the chemical composition of the basic elements of the cell walls of these bacteria. Although cross-reacting antigens very easily are demonstrated by simple gel precipitin tests, this method has not been sufficiently exploited as a complement to the chemical analysis. This might be due to the fact that this type of serological analysis has turned out to be difficult to standardize if applied to antigens from enteric bacteria. By careful evaluation of methods for the preparation of immunizing antigens good antisera can regularly be obtained. The fact, that the qualitative and quantitative responses to one antigen vary widely in different rabbits (*Carlisle et al.* 1962) need not be a drawback in this context. On the contrary, by using individually different antisera with suitable characteristics the sensitivity of the gel precipitin test may be increased.

[3] Prepared in collaboration with Dr. R. Kopp, Hygiene Institut der Universität, Freiburg i. Br.

SUMMARY

Experiments have been performed to evaluate suitable conditions for the analysis of antigenic components from cell walls of enteric bacteria by gel precipitin tests Cell wall preparations from UV-killed bacteria were found to be superior to other antigens for the immunization of rabbits for the production of antisera A convenient method for the preparation of precipitating antigens was the sonic tratment of heat killed cells

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THE STABILITY OF LEUCOCYTES IN URINE INFECTED WITH PROTEUS

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Received 10.vi.65

During the past decades bacteria belonging to the genus Proteus have become increasingly often isolated from clinical specimens, probably owing to selection of bacteria more resistant to chemotherapy. Proteus is now particularly often isolated from the urine of patients with signs of urinary tract infection (*Dorlen* 1950, *Edebo & Laurell* 1958). It has been observed (*Sanford* 1956, *Edebo & Laurell* 1958), however, that although Proteus was isolated in great numbers, the occurrence of leucocytes was relatively sparse and irregular, and on some occasions no leucocytes could be demonstrated. *Sanford* (1956) also pointed out that in roughly one third of cases the pH of the urine was 7.0 or higher.

One characteristic property of the most commonly encountered strains of the genus *Proteus*, *P. mirabilis* and *P. vulgaris*, is the rapid production of large amounts of urease (*Rustigian & Stuart* 1945). Urease catalyses the decomposition of urea into carbon dioxide and ammonia which leads to increasing alkalinity of the medium. It has been demonstrated in experimental pyelonephritis in rats and in cultures of monkey kidney cells (*Braude et al.* 1960) that the caracteristic pathogenicity of Proteus is dependent on the organism's capacity to split urea. In the presence of urea Proteus grows in the tubular cells, this leads to an increase in pH and eventually to necrosis of the cells. Similar but sterile cell injuries could be produced by urease.

It is known (*Tullis* 1953, *Walford* 1960) that the stability of leucocytes in alkaline suspensions is poor. There are thus two possible explanations of the scarcity of leucocytes in Proteus infected urine, 1) few or no leucocytes are released into the infected urine, 2) leucocytes are released into the urine but later disintegrate, supposedly owing to a rise in pH. The experiments described below were carried out to test the latter part of the second hypothesis, i.e. whether leucocytes disintegrate in urine infected with *Proteus*.

MATERIAL AND METHODS

Bacteria cultivated for 18 hours in nutrient broth (Difco) were inoculated into nutrient broth with urea added in concentrations from 0 to 2 per cent The follow ing bacteria were tested *P vulgaris* (the X19 strain), *P mirabilis Alcaligenes fae calis Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* All the cell incubations described in this paper were done at 37° C

Because of scarcity of material when the leucocytes were investigated the hy drogen ion concentration during the course of the experiments was estimated with pH paper (Universal Indikatorpapier, Merck AG, Darmstadt) The pH at the con clusion of the experiment was measured with a pH-meter (pH meter 22 Radio meter, Copenhagen)

Leucocytes were prepared in accordance with the directions of *Szilard* (1926) 30 ml of 3 per cent sodium citrate was added to 100 ml of sheep blood and 100 ml of a mixture consisting of 4 parts of 2 5 per cent acetic acid and 1 part of 2 per cent tartaric acid was added The flask with this mixture was gently rotated for 30 to 45 seconds This treatment, which lowered the pH to about 2 caused total lysis of the red blood cells, but the leucocytes suffered little harm Potassium hydroxide (2 per cent) was then added to neutral reaction (in our experiments 140 ml potas sium hydroxide per 100 ml acid mixture) The fluid was then centrifuged for 10 minutes at approximately 90 xg (International refrigerated centrifuge model PR 2 rotor 16 cm 700 r p m), the supernatant was drawn off and the remaining leuco cytes were washed twice in a preserving fluid The preserving fluid had the follow ing composition (*Ruthberg & Terenteva* 1959) glucose 0 4 g gelatin 3 5 g ascorbic acid 0 3 mg, Na_2HPO_4 12 H_2O 16 mg KH_2PO_4 8 mg K_3PO_4 3 mg NaCl 500 mg, KCl

water to 100 ml

stimated after counting more than 300 cells in a

lso followed with a colorimeter (Beckman C, red filter = 660 mμ)

The influence of bacteria on leucocytes was investigated by incubating bacteria in leucocyte suspensions The suspension was made up of 12 ml of a leucocyte (10^8 cells/ml) suspension in preserving fluid, 36 ml nutrient broth and urea at a final concentration of one per cent This suspension was divided into samples of 4 ml, and 0 2 ml of an overnight culture of the bacteria in nutrient brot was added

The influence of bacteria on leucocytes when direct contact between the different kinds of cell was excluded was tested by growing bacteria in dialysis bags immersed in the leucocyte suspensions Cellophane dialysis tubings (diameter 6 35 mm) were knotted at one end The other end was provided with a piece of glass tubing immersed in water, and sterilized by autoclaving The dialysis tubings were filled with 4 ml of nutrient broth with 1 per cent urea added different bacteria were introduced, and the tubings lowered into suspensions of leucocytes in preserving fluid

Human urinary leucocytes were tested by collecting urine from a patient with urinary tract infection due to *E coli* of a strain sensitive to penicillin (2 IU/ml) and streptomycin (16 mcg/ml) To approximately 50 ml of urine 50 000 IU of penicillin and 0 25 g of streptomycin sulphate were added Strains of the original *E coli* and *P vulgaris* made resistant to penicillin and streptomycin were inoculated into separate samples and incubated at 37° C The minimum inhibiting concentra tions were for the resistant strains more than 10 000 IU of penicillin per ml and more than 5 mg of streptomycin per ml Urine with no resistant strains added was incubated at the same time

RESULTS

Strains of *P vulgaris P mirabilis, Alc faecalis, E coli, Ps aerugi nosa*, and *Staph aureus* were inoculated into series of nutrient broth tubes containing different concentrations of urea The pH values me asured in broth with 1 per cent urea are given in Fig 1 At concentra tions below 0 1 per cent the pH was little affected by *Proteus*, and at concentrations above 0 5 per cent the results were essentially the same

Signs of Figures

- ○ No bacteria
- No bacteria no urea
- *Str faecalis*
- ● *Staph aureus*
- □ *P mirabilis*
- *P vulgaris* with 1 per cent urea added
- *Alc faecalis*
- ■ *P vulgaris*
- ◇ *Ps aeruginosa*
- *E coli*
- *Aerob aerogenes*
- ◆ *E coli*

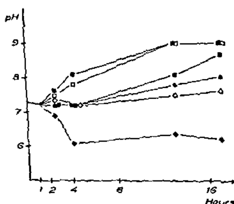


Fig 1
Effect on pH by inoculation of different bacteria into nutrient broth containing 1 per cent urea

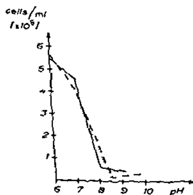


Fig 2
Number of leucocytes at different pH levels after 1 (———), 3 (— — —), and 10 ( ) hours

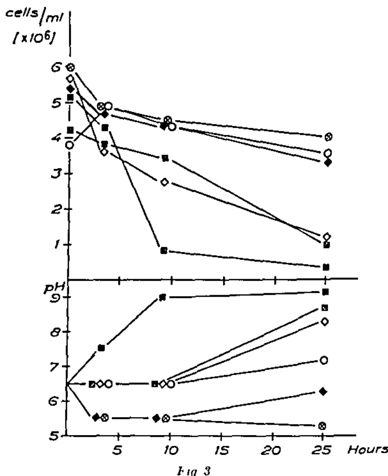


Fig 3

Influence of different bacteria on pH and number of leucocytes when grown in nutrient broth containing 1 per cent urea

as for 1 per cent *Proteus* caused the most rapid rise in pH After 4 hours the pH was about 8, and after 13 hours it had reached a level of about 9 *E coli* caused a rapid fall in pH The remainder of the strains tested did not affect the pH markedly during the earlier part of the experiment Later Alcaligenes in particular caused a considerable increase in pH

The stability of leucocytes at different pH levels was tested in borax—boric-acid and ammonia—borax—boric-acid buffers Leukocytes suspended in preserving fluid were added to the buffers to give a pH range of 6–9 5 The leucocytes were counted after 1, 3, and 10 hours After 1 hour at pH 8 about 90 per cent of the leucocytes had already disintegrated (Fig 2) Most of the remaining cells were swollen and had swollen nuclei After 10 hours at pH 6 more than 80 per cent of the cells were left apparently undamaged, at pH 8 5 less than 1 per cent remained At pH 8 0 and above viscous aggregates were formed, consisting of tangles of filaments with granules, several leucocyte ghosts, and a few morphologically intact leucocytes embedded in the meshes

Suspensions of leucocytes in a mixture of preserving fluid and broth

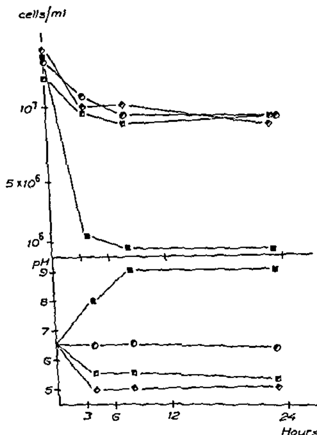


Fig 4

Effect of *P vulgaris* and *E coli* upon pH and leucocyte count in nutrient broth

with 1 per cent urea (see methods) were inoculated with *P vulgaris*, *Alc faecalis* *E coli*, *Ps aeruginosa*, and *Str faecalis* (Fig 3) The pH in the tube infected with *Proteus* increased concomitantly with the fall in the leucocyte count During the first 9 hours the pH increased from 6 5 to 9 0 and the leucocyte count decreased from 5 1 10^6/ml to 8 5 10^5 ml A slower but considerable increase in leucocyte concentration was produced by *Alc faecalis* and *Ps aeruginosa* *E coli* and *Str faecalis* lowered the pH, and did not significantly affect the number of leucocytes compared with the blank

When *P vulgaris* and *E coli* were inoculated into broth containing leucocytes but no urea the pH was little affected, and there was little effect on the number of leucocytes (Fig 4)

When the bacteria were cultivated in dialysis bags immersed in leucocyte suspensions the pH and optical desity of the leucocyte suspensions were affected (Fig 5) After 4 hours the pH in the *P vulgaris* and *P mirabilis* specimens had increased to 8 1 The light transmission

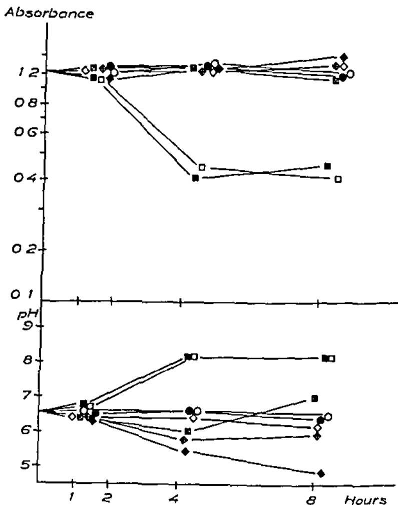


Fig 5
Influence of different bacteria on pH and optical density (absorbance 660 mμ) when grown in dialysis bags immersed in nutrient broth containing leukocytes and 1 per cent urea

of the leucocyte suspensions surrounding the Proteus cultures had also increased The other bacterial species caused only minor changes in pH and optical density A similar experiment run with *P vulgaris*, in which the leucocytes were estimated by counting, gave essentially similar results

Penicillin and streptomycin were added to urine from a patient with a urinary-tract infection due to *E coli* in order to kill these bacteria Later *P vulgaris* and *E coli* resistant to these antibiotics were inoculated (Fig 6) Proteus raised the pH and reduced the leucocyte concentration, whereas *E coli* had little effect

DISCUSSION

Proteus strains cause a rapid increase in pH in urea-containing media This is probably due to the abundant production of urease,

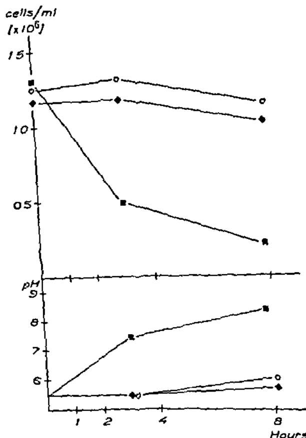


Fig 6

The effect of *P vulgaris* and *E coli* on pH and number of leucocytes in urine from a patient with urinary tract infection

since urease splits urea into ammonia and carbon dioxide, which raises the pH The stability of leucocytes is conspicuously reduced at pH values above 8 0 When Proteus creates alkalinity the leucocytes are disintegrated The behaviour of the leucocytes in infected suspensions is dependent on pH, just as in sterile suspensions When the bacteria were separated from the leucocytes by a cellophane membrane the pH rose in the leucocyte suspension, probably owing to diffusion of hydrogen and hydroxyl ions Concurrently with the pH rise the leucocytes became lysed Since the cellophane membrane did not permit diffusion of larger molecules, no direct enzyme action can have been responsible for the leucocyte disintegration Leucocytes obtained from bacterially infected urine behaved essentially in the same way as leucocytes prepared from blood

Alc faecalis and *Ps aeruginosa* produced a slower increase in pH and correspondingly slower leucocyte disintegration *E coli* and *Str*

faecalis produced a more acid medium in which the leucocytes were stable.

This investigation was initiated by the clinical observation that often when Proteus was present in human urine, few leucocytes could be demonstrated. This might be due to the fact that few leucocytes ever entered the urine. However, if leucocytes did enter the urine, they would be expected to disintegrate, since Proteus rapidly makes the urine alkaline. Our results indicate that the latter hypothesis is possible. The disintegration may occur both in the urine bladder, and in voided urine. It remains, however, to be demonstrated that leucocytes do indeed enter the urine in urinary tract infections due to Proteus and subsequently disintegrate.

SUMMARY

The stability of leucocytes in Proteus-infected urine or other urea media is low. This may be attributed to the alkalinity due to the formation of urease by Proteus. One reason for the scantiness of leucocytes in urinary specimens from patients with urinary-tract infections may consequently be alkaline disintegration of exudated cells.

Alc. faecalis and *Ps. aeruginosa* have a similar but slower and less pronounced effect. Whether or not this effect is of practical importance is to be further investigated.

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EXTREMELY HIGH CONTENT OF ANTI-α-STAPHYLOLYSIN IN SERUM SAMPLES FROM A PATIENT WITH MULTIPLE MYELOMA

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Sera from patients with multiple myeloma generally contain only small amounts of isoagglutinins (7), and antibodies such as anti-streptolysin and anti-staphylolysin are found less frequently than in normal sera (7, 11). Patients with multiple myeloma *do not react to an antigen* stimulus to the same extent as normal individuals (4). This has lent some support to the concept of myeloma proteins as incomplete γ-globulins (7, 10). Recently, however, *Burnet* (2) put forward a hypothesis for the origin of the myeloma cells. According to this hypothesis these cells should arise due to a clonal proliferation of a mutated plasma cell. There is indeed some reason to believe that the M component, *produced* by plasma cells in patients with myeloma and other "*M diseases*", consists of only one or a few closely related γ-globulins (2, 12). This is further supported by the demonstration of extremely high anti-streptolysin titres in patients whose sera contain an M component which is *thought to* possess the anti-streptolysin activity (5, 1, 12, 13).

The present paper is concerned with the demonstration of an extremely high anti-α-staphylolysin content *in serum samples* from a patient with multiple myeloma, and with some *experiments* which were carried out in order to further characterize the *myeloma* protein by correlating the serological experiments with physico-chemical methods.

MATERIAL AND METHODS

The myeloma serum originated *from a patient* suffering from myelomatosis which was diagnosed on the basis of typical clinical and laboratory findings. The bone marrow obtained by sternal *puncture* was found to contain two per cent plasma cells. X-ray examination of the bones revealed numerous typically myelomatous foci. The sample used for most *of the investigations* was obtained in August 1964. A few drops of a sample obtained in 1962 and stored since then at −20° C were

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We would like to thank Professor *Aage Videbæk*, M.D., Copenhagen County *Hospital* Gentofte, for the supply of patient serum and for permission to publish the clinical data. The skilful technical assistance of Mrs. *Tove Porst* is gratefully acknowledged.

used for comparison It should be mentioned that the patient had been treated with the cytotoxic drug Melphalan since 1962

Other serum samples from the routine test for anti-α staphylolysin were chosen as controls for the different analyses The selected sera included some with increased titres (≧ 8, see below) as well as some showing a titre < 2

Titration of anti α staphylolysin was performed according to the routine technique used in the Streptococcal Department of this Institute (6) The titration is carried out in liquid medium and the antibody titres are given in units per ml with reference to the international standard[1] In addition a titration of the sera was carried out in solid medium The technique employed was as follows A melted phosphate buffered (pH 7 38) agar solution (three per cent) was mixed at 48° C with an equal volume of a ten per cent suspension of washed rabbit red cells suspended in the same buffer Approximately 2 mm thick blood agar plates were prepared in Petri dishes by pouring the melted agar (1 5 per cent agar and 5 per cent red cells) The solidified plates were stored overnight before use 4 mm holes were cut in the blood agar and filled with serum Saline was used for controls After one hour at room temperature when the samples were drained into the agar, the holes were filled with α staphylolysin (0 3 U/ml) A lytic zone developed concentrically to the holes filled with saline while anti α staphylolysin containing sera inhibited the lysis Inhibition could be demonstrated when sera diluted to a content of as little as one unit of antibody per ml were applied

Immunoelectrophoretic Analysis

Scheidegger's micromodification of the original technique with the previously described minor modifications (8) was employed The following rabbit antisera were used A polyvalent anti human serum and specific antisera against the human immune globulins i e anti γG anti γA and anti γM (3)

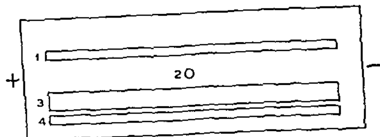
Combined Technique of Haemolysis Inhibition and Immunoelectrophoresis (HII)

This technique was developed in order to correlate an immunoelectrophoretic analysis with a determination of the mobility of the anti-α staphylolysin The following modifications of our immunoelectrophoretic technique were introduced (Fig 1) After electrophoresis of the serum sample a 4 × 65 mm trough was cut 2 mm from the central hole 1 5 per cent agar containing 5 per cent rabbit erythrocytes as described above was poured into the trough Next a 2 mm wide trough was cut between the edge of the slide and the blood agar at a distance of one mm from the latter This trough was filled with 0 1 ml α staphylolysin (0 3 U/ml) Finally a third trough was cut on the opposite side of the central hole and this trough was filled with anti human serum—as in the conventional technique—for the development of the immunoprecipitation arcs A progressive lysis of the red cells occurred due to the gradual diffusing of lysin except where the cells were protected by α staphylolysin antibodies The plates were read after a diffusion time of 20 24 hours

Paper electrophoresis was carried out using TRIS/EDTA/borate buffer (8) To determine the electrophoretic mobility of the anti α staphylolysin on the paper a strip was cut in 1 cm pieces immediately after electrophoresis Each of the 13 paper segments obtained were eluted in tubes containing 0 5 ml phosphate buffered saline (pH 7 38) The tubes were shaken frequently for 5 hours and kept overnight at 4° C The eluates were used for titration and HII experiments

Moving boundary electrophoresis according to *Tiselius* was employed for obtaining 0 3 ml fractions of various composition (see text Fig 4) The serum of the patient was diluted to a 1 5 per cent concentration of total protein and the technique described by *Marner* (9) was used for drawing off the fractions Four and five fractions were withdrawn from the ascending and descending limbs respectively In addition the solution contained in the bottom section of the electrophoresis cell was withdrawn and used together with the other fractions for titration and HII experiments

1 One unit of α staphylolysin is defined as the amount of lysin which is partially inhibited by one unit of standard serum



1 ANTI HUMAN SERUM
2 SERUM SAMPLE
3 BLOOD AGAR
4 α-STAPHYLOLYSIN

Fig 1

plied in 2 rabbit blood agar is poured into trough No 3 Finally, troughs Nos 1 and 4 are filled with anti human serum and α staphylolysin respectively

RESULTS

The anti α-staphylolysin titre of the serum obtained from the patient in 1961 was repeatedly found to be 800, whereas the sample obtained in 1962 and stored at −20° C had a titre of 6400 Both titres are extremely high compared to the titres (8–24, rarely 32) of sera from patients with staphylococcal infections

A series of serum samples from the routine investigation as well as the standard serum were titrated for anti-α staphylolysin in blood agar plates Despite slight differences in the diameter of the haemolytic zone of the controls on different days, reproducible titres were obtained The titres of the myeloma serum samples, as determined by this technique, were of the same magnitude as those obtained in liquid medium

Immunoelectrophoretic analysis of the serum of the patient indicated the presence of an M component of γG antigenic character with γ_1 mobility

An example of an HI experiment is shown in Fig 2 C The undiluted sample applied had a titre of 12 units ml The zone of inhibition has a lengthwise distribution similar to the mobility range for γG globulin A few samples among the sera used as controls showed a double curved zone of inhibition within the immune globulin mobility range This is presumably due to the presence of anti-α staphylolysin antibod...

... demonstrated inhibition when 2 µl were applied

The serum ... ent was analysed at different dilutions When

used for comparison It should be mentioned that the patient had been treated with the cytotoxic drug Melphalan since 1962

Other serum samples from the routine test for anti-α staphylolysin were chosen as controls for the different analyses The selected sera included some with increased titres (≧ 8, see below) as well as some showing a titre < 2

Titration of anti α staphylolysin was performed according to the routine technique used in the Streptococcal Department of this Institute (6) The titration is carried out in liquid medium and the antibody titres are given in units per ml with reference to the international standard [1] In addition a titration of the sera was carried out in solid medium The technique employed was as follows A melted phosphate buffered (pH 7 38) agar solution (three per cent) was mixed at 46° C with an equal volume of a ten per cent suspension of washed rabbit red cells suspended in the same buffer Approximately 2 mm thick blood agar plates were prepared in Petri dishes by pouring the melted agar (1 5 per cent agar and 5 per cent red cells) The solidified plates were stored overnight before use 4 mm holes were cut in the blood agar and filled with serum Saline was used for controls After one hour at room temperature when the samples were drained into the agar the holes were filled with α staphylolysin (0 3 U/ml) A lytic zone developed concentrically to the holes filled with saline while anti α staphylolysin containing sera inhibited the lysis Inhibition could be demonstrated when sera diluted to a content of as little as one unit of antibody per ml were applied

Immunoelectrophoretic Analysis

Scheidegger's micromodification of the original technique with the previously described minor modifications (8) was employed The following rabbit antisera were used A polyvalent anti human serum and specific antisera against the human immune globulins, *i e* anti γG, anti-γA and anti γM (3)

Combined Technique of Haemolysis Inhibition and Immunoelectrophoresis (HII)

This technique was developed in order to correlate an immunoelectrophoretic analysis with a determination of the mobility of the anti-α staphylolysin The following modifications of our immunoelectrophoretic technique were introduced (Fig 1) After electrophoresis of the serum sample a 4 × 65 mm trough was cut 2 mm from the central hole 1 5 per cent agar containing 5 per cent rabbit erythrocytes as described above was poured into the trough Next, a 2 mm wide trough was cut between the edge of the slide and the blood agar at a distance of one mm from the latter This trough was filled with 0 1 ml α staphylolysin (0 3 U/ml) Finally a third trough was cut on the opposite side of the central hole and this trough was filled with anti human serum—as in the conventional technique—for the development of the immunoprecipitation arcs A progressive lysis of the red cells occurred

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(8) To de

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strip was cut in 1 cm pieces immediately after electrophoresis Each of the 13 paper segments obtained were eluted in tubes containing 0 5 ml phosphate buffered saline (pH 7 38) The tubes were shaken frequently for 5 hours and kept overnight at 4° C The eluates were used for titration and HII experiments

Moving boundary electrophoresis according to *Tiselius* was employed for obtaining 0 3 ml fractions of various composition (see text Fig 4) The serum of the patient was diluted to a 1 5 per cent concentration of total protein and the technique described by *Marner* (9) was used for drawing off the fractions Four and five fractions were withdrawn from the ascending and descending limbs respectively In addition the solution contained in the bottom section of the electrophoresis cell was withdrawn and used together with the other fractions for titration and HII experiments

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1 One unit of α staphylolysin is defined as the amount of lysin which is partially inhibited by one unit of standard serum

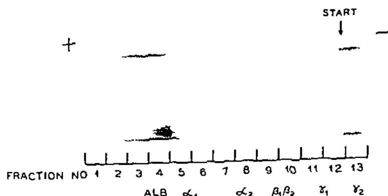


Fig 3

shows the result of paper electrophoretic analysis of the patient serum sample from 1964 This paper strip was obtained within the same electrophoretic run as that used for cutting and elution as indicated on the figure While eluates Nos 1 11 contained < 2 units/ml No 12 and No 13 titrated 4 and 12 units of anti α staphylolysin per ml respectively

undiluted serum was applied an unsymmetric inhibition zone was found with maximum width just opposite the center of the myeloma protein precipitation line and tapering off in the direction of the cathode (Fig 2 A) With serum diluted 25 times or more a symmetric inhibition zone was seen (Fig 2 B) A zone was just visible when a dilution 1 2000 (the 1964 sample) and 1 5000 (the 1962 sample) was analysed Thus, due to the electrophoretic homogeneity of the antibody, even dilutions containing 1 3 unit ml caused a zone of inhibition

TABLE 1

Fraction No	Electrophoretic components	Titre (units ml)	Inhibition by HII analysis
1	Alb α_1	< 2	not done
2	Alb α_1 α_2	< 2	0
3	Alb α_1 α_2 β_1 β_2	< 2	0
4	Alb α_1 α β_1 β γ_1	> 3 < 6	(+)
5	[illegible]	> 3 < 6	(+)
6	[illegible] γ_2	50	+
7	[illegible] γ_2 γ_1	100	+
8	[illegible] γ_2 γ_1 β_2 β_1	100	+
9	[illegible] γ_2 γ_1 β_2 β_1 α_2	100	+
10	All (bottom compartment)	> 100 < 128	+

[illegible] ults of the M component (—

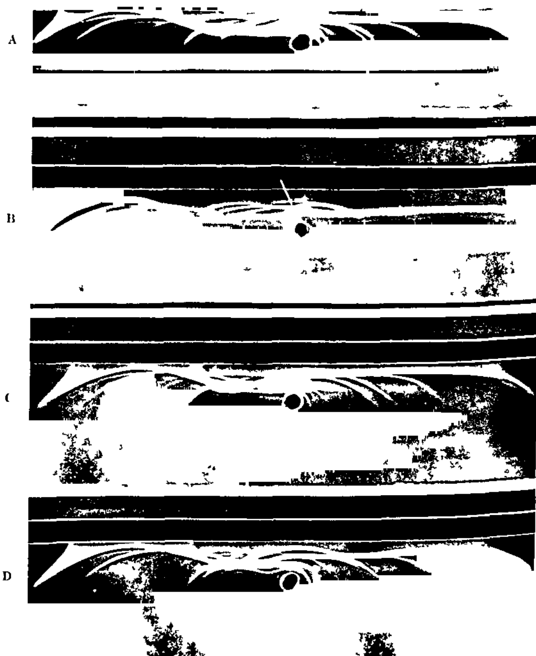


Fig 2

Four examples of analysis by the HII technique are shown

A A serum sample from the myeloma patient titre 800 units/ml

B The same serum sample diluted 25 times

C A serum sample from a patient with a chronic ulcer of the leg The titre of this serum is 12 units/ml

D A serum sample with a titre < 2 units/ml

The titres stated are determined in liquid medium

The inhibition zone at A is clearly elongated cathodically whilst the zone at B faces the precipitation arc of the M component (indicated by an arrow) At C the zone is distributed lengthwise in the same mobility range as for γG globulin Inhibition could not be demonstrated by analysis of a serum which had a titre < 2 units/ml (compare Fig 2 D)

Fig 4 The different positions for the M component with respect to other serum proteins in immunoelectrophoresis versus paper and free electrophoresis are presumably due to the different types of supporting media

The titres of the eluates from the paper electrophoresis and of the fractions from moving boundary electrophoresis are shown in the legend for Fig 3 and in Table 1, respectively These results also indicate that the anti α staphylolysin has the same rate of migration as the M component

The eluates Nos 12 and 13 from the paper electrophoresis experiment developed an inhibition in HII test similar to that obtained with genuine serum i e an inhibition zone of maximum width close to the point of application

The fractions obtained from moving boundary electrophoresis were also submitted to HII The results were in agreement with the anti α staphylolysin titres Fraction 4 and 5 showed an inhibition zone which was just discernible Fraction 6–9, however, showed very strong inhibition (Fig 4 and Table 1) The zones were more elongated than those obtained with genuine serum but always with the maximum facing the M line The reason for this elongation is unknown The storage for about four months at 4° C prior to the HII experiments might be responsible for the altered appearance of the inhibition zone

DISCUSSION

Two serum samples obtained in 1962 and 1964 from a *myeloma* patient showed extremely high anti-α staphylolysin titres, 6400 and 800 *units*/ml respectively This unusual finding raised the question whether the inhibitory effect is concerned with the M component or not This question was elucidated by the use of a modified agar electrophoretic technique HII which combines haemolysis inhibition with immuno electrophoresis It is possible to determine the mobility of anti-α staphylolysin simultaneously with immunoelectrophoretic analysis by means of this procedure The sensitivity and the reproducibility of the HII method was established by HII analysis of control samples It should be emphasized that the maximum width of the inhibition zone which is obtained by analysis of undiluted positive sera is mainly determined by the diffusion rate of the α staphylolysin and not of the titre of the sample

All the results of HII analysis of genuine or electrophoretically fractionated samples of serum from the patient presented evidence that the myeloma protein is responsible for the high content of anti-α staphylolysin These experiments do not however answer the question whether the inhibitory effect is due to a true antibody or to some type of unspecific reaction

Recently a systematic analysis of serum samples from patients with

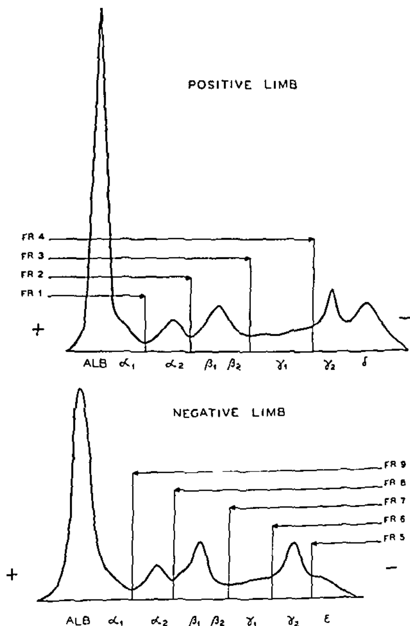


Fig 4

The curves obtained from the positive and negative limb by moving boundary electrophoresis of the serum from the patient show an M component with γ_2 mobility. The arrows indicate the position of the syringe during the withdrawal of the individual fractions. The titres of each fraction are listed in Table 1 together with the results of the HII analysis

Paper electrophoretic analysis of the serum samples was carried out simultaneously and revealed an M component of slow mobility (Fig 3). The concentration of the M component was calculated to be 13.6 and 34.1 per cent of the total proteins for the samples withdrawn in 1964 and 1962, respectively. A similar result was obtained by moving boundary electrophoresis of the sample from 1964 (13.0 per cent γ_2-globulin).

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STUDIES ON ANTILIPOIDAL IMMUNE GLOBULINS

3 *Thermal Stability of the Antilipoidal Immune Globulins Found at Various Stages of the Syphilitic Infection and in Non-Syphilitic Reactors*

By

Erik Holst and Michael Weis Bentzon

Received 7 III 65

It has been demonstrated previously that heat inactivation for 30 minutes at 56° C usually results in a reduction of the titre of sera which react in the complement fixation test with ordinary cardiolipin antigen (CL C)

The same is true of reactions with the lecithin-free cardiolipin antigen cardchol (CC), the reduction in titre in this case being usually more pronounced than with CL C antigen (6)

This reduction in reactivity might be due to a thermal effect either on the immune globulins involved in the lipoid-antilipoidal reaction or on other serum factors

Investigations previously reported by this laboratory demonstrated that in all probability the reduction in titre registered after heat inactivation reflects a direct thermal influence upon the antilipoidal immune globulins (2)

Differences between various antilipoidal sera and other body fluids with regard to reduction in reactivity following heat inactivation would thus indicate differences in the heat stability of the antilipoidal im-

... ine the thermal sta ... well as with CC an- ... number of sera and cerebrospinal fluids (CSF) representing the various stages of the syphilitic infection and in sera from non-syphilitic reactors

MATERIAL AND METHODS

... been adhered to

myelomatosis was initiated in order to establish the frequency with which high anti-α-staphylolysin titres could be found. Strange to say, but within the first series of 30 samples, a γA myeloma patient whose serum contained 6400 units of anti-α-staphylolysin per ml, was encountered. Further investigation of the sera of the two patients now available is needed before a more detailed characterization of the inhibitor(s) can be given.

SUMMARY

An unusually high concentration of anti-α-staphylolysin was found in serum samples from a myeloma patient (the 1962 sample contained 6400 units/ml and the 1964 sample 800 units/ml). Serum fractions obtained by electrophoretic methods demonstrated that the anti-α staphylolysin activity was associated with the M component.

A method, HII (*h*aemolytic *i*nhibition and immunoelectrophoresis), which permits correlation of the mobility of anti-α-staphylolysin with immunoelectrophoretic analysis, was developed and its limitations in providing evidence for a true antibody against α-staphylolysin being associated with the M component is discussed.

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with CC antigen, the maximum mean titres in both cases representing the results obtained with sera from cases of secondary syphilis

A preliminary statistical analysis justified collection of the original five diagnostic groups into three main groups, each characterized by a mean reduction in titre following heat inactivation differing significantly from that found in the two other groups These three main groups are *Early syphilis* ($S_{I \& II}$), comprising primary and secondary syphilis *Late syphilis* ($S_{III \& LT}$), comprising tertiary and late latent syphilis, and *Non syphilitic reactors* (Non syph)

TABLE 2

Effect of Heat Inactivation (56° C/30 Min) on the Reactivity in Complement Fixation Test with Cardiolipin (CLC) Antigen Expressed as Mean Differences in $\log_{10}$ Titre Value between Unheated and Heat Inactivated Serum for 179 Antilipoidal Sera Grouped According to Degree of Reactivity within each of three Diagnostic Groups

Group	No	$\log_{10}$ titre difference unheated heat-inact	s^2	SE
$S_{I \& II}$ low	25	0.115	0.0[illegible]41	0.028
$S_{I \& II}$ high	70	0.131	0.0188	0.017
$S_{I \& II}$ -	95	0.127	0.0200	0.015
$S_{III \& LT}$ low	27	0.259	0.0592	0.040
$S_{III \& LT}$ high	18	0.204	0.0193	0.048
$S_{III \& LT}$	45	0.237	0.0434	0.031
Non syph low	22	0.519	0.1019	0.071
Non syph high	17	0.373	0.1250	0.081
Non syph	39	0.456	0.1119	0.054

Table 2 gives the mean reduction in $\log_{10}$ CLC titre values following heat inactivation calculated for each of the three main groups with standard deviation and standard error Within each group separate calculations of mean titre reductions, standard deviation and standard error were made in order to reveal possible differences between sera with mean titres from 0.5 to 1.0 $\log_{10}$ value (low) and sera with mean [illegible] seen that there is no [illegible] s registered for sera

[illegible] also be seen that the mean titre reduction following heat inactivation is significantly smaller in the $S_{I \& II}$ group than in both the $S_{III \& LT}$ and Non S groups, and that the reduction in mean titre in the $S_{III \& LT}$ group is significantly smaller than that found in the Non S group

The results with CC antigen are shown in Table 3 The mean titre reductions following heat inactivation are more pronounced with this antigen for both the $S_{I \& II}$ and $S_{III \& LT}$ groups than with CLC antigen and the standard deviations are higher However, the reduc

Primary syphilis (S I) Typical clinical lesion and demonstration of *Treponema pallidum* (T p) and/or T p immobilizing antibodies

Secondary syphilis (S II) Typical clinical lesion and demonstration of T p immobilizinz antil odies with or without demonstration of T p

Tertiary syphilis (S III) Typical neurological or cardiovascular lesions and de m

of antilipoidal ar without history of

ig for more than three months without history of syphilitic infection without clinical symptoms of syphilitic infection and without T p immobilizing antibodies

Thus the study includes 32 sera fr of secondary syphilis 20 sera and 7 cases of late latent syphilis and 48

All sera and CSFs were examined within three days of withdrawal of the sample without preliminary storage or refrigeration Unheated and heat inactivated aliquots of the same sample were examined on the same day and with both antigens

The antigens used in the complement fixation tests were (a) Ordinary cardiolipin antigen (ClC) containing 0 0175 per cent (w/v) cardiolipin 0 0875 per cent (w/v) egg lecithin (both prepared at Statens Seruminstitut according to *Pangborn* (4)) and 0 3 per cent (w/v) cholesterol (Pfanstiehl) in dehydrated ethanol and (b) the lecithin free cardiolipin antigen cardchol (CC) containing 0 0175 per cent (w/v) cardiolipin and 0 3 per cent (w/v) cholesterol in dehydrated ethanol

The technique for quantitative examination with these two antigens has been described previously (2 5) The results are given as $\log_{10}$ titre value calculated according to *Karber* (1) The CSFs were examined with ClC antigen only using the technique described for serum examination but a CSF dose of 0 125 ml *i e* five times the amount of serum used in this technique

RESULTS

Mean $\log_{10}$ titre values calculated for unheated and heat inactivated sera in each diagnostic group are shown separately for each antigen in Table 1 It will be seen that the mean titres for unheated sera are of comparable magnitude for all diagnostic groups, varying from 1 039 to 1 424 $\log_{10}$ value with ClC antigen and from 0 945 to 1 391 $\log_{10}$ value

TABLE 1

Mean $\log_{10}$ Titre Values in Complement Fixation Test with Cardi lipin (ClC) and with Cardchol (CC) Antigen for 191 Antilipoidal Sera Grouped According to Clinical Diagnosis (Sera Examined Unheated as well as after Heat Inactivation (56° C/30 min))

	Diagnosis	No	Mean $\log_{10}$ titre ClC		Mean $\log_{10}$ titre CC	
			Unheated	Heat inact	Unheated	Heat inact
1	S I	32 26	1 174	1 052	1 208	0 960
2	S II	63 56	1 424	1 295	1 391	1 204
3	S III	17 11	1 228	0 995	0 950	0 395
4	S LT	28 15	1 039	0 799	0 945	0 426
5	Non syph reactors	3[illegible] 37	1 137	0 683	1 172	0 638

S $_{I \& II}$ group and significantly smaller than the mean reductions calculated fo both the S $_{III \& IT}$ and Non-syph group, in spite of the fact that these CSFs originated from cases of tertiary syphilis

DISCUSSION AND CONCLUSIONS

It would seem natural to conclude that various forms of immune globulins characterized by different heat stability are responsible for antilipoidal reactivity The results of this study might be explained by the existence of three different forms of antilipoidal immune globulins with different heat stability. one responsible for the antilipoidal reactivity in CSF and in early syphilis, the two others responsible for the antilipoidal reactivity in late syphilis and in non syphilitic reactors, respectively It seems more natural to assume that there are two (or more) forms of immune globulins differing in heat stability, both (or all) of which may be present in all the conditions under study, and that the differences in heat stability registered are due to variations in the relative amounts of these two (or more) forms of antilipoidal immune globulins

In any event, it must be concluded from these observations that at least two forms of antilipoidal immune globulins differing in heat stability are produced in the course of the syphilitic infections the more heat stable immune globulins dominating in serum from cases of primary an ... ertiary syphil ... in serum f ...

The ... by a heat stability inferior to that of syphilitic antilipoidal antibody This represents either a totally different immune globulin or, more likely, a preponderance of, or even a pure form of the less heat ...

... with CLC antigen however there is close agreement between the results obtained with CC and CLC antigens as far as the non-syphilitic sera are concerned

The discrepancy between the results obtained with these antigens would seem to lend support to the idea that CLC and CC antigens react with antilipoidal immune globulins which differ from each other The present results may, however, also be explained ... ence ... tow ... more ... immune globulins and CC more sensitive to less heat-stable immune globulins

The latter concept would be consistent with earlier findings concerning the influence of lecithin on the sensitivity of cardiolipin antigens (3)

tion in mean titre for non-syphilitic reactors found with CC antigen does not exceed that found with CLC antigen

The S $_{I \,\&\, II}$ group showed a significantly smaller mean titre reduction than both the S $_{III \,\&\, IV}$ and Non-syph groups, but contrary to the findings with CLC antigen, with CC antigen there was no significant difference between the mean titre reduction registered for the S $_{III \,\&\, IV}$ and Non-syph groups

TABLE 3

Effect of Heat Inactivation (56° C/30 Min) on the Reactivity in Complement Fixation Test with Cardchol (CC) Antigen Expressed as Mean Difference in log_{10} Titre Value between Unheated and Heat Inactivated Serum for 145 Antilipoidal Sera Grouped According to Degree of Reactivity within each of three Diagnostic Groups

Group	No	Log_{10} titre difference unheated heat inact	s^2	SE
S I & II low	22	0.249	0.0252	0.032
S I & II high	60	0.189	0.0204	0.019
S I & II	82	0.205	0.0221	0.016
S III & IV low	20	0.593	0.0568	0.054
S III & IV high	6	0.336	0.0164	0.099
S III & IV	26	0.534	0.0586	0.047
Non syph low	26	0.537	0.0502	0.044
Non syph high	11	0.324	0.0197	0.067
Non syph	37	0.474	0.0501	0.037

TABLE 4

Effect of Heat Inactivation (56° C/30 Min) on the Reactivity in Complement Fixation Test with Cardiolipin (CLC) Antigen for 7 Antilipoidal Cerebrospinal Fluids from Cases of Tertiary Syphilis

CSF no	Log_{10} CLC titre unheated	Log_{10} CLC titre heat inactivated	Log_{10} titre difference unheated heat inact
1	0.716	0.716	0.000
5	0.692	0.620	0.072
6	1.241	1.288	−0.047
7	1.575	1.575	0.000
8	1.050	0.907	0.143
9	0.477	0.334	0.143
27	1.193	1.193	0.000
Mean	0.992	0.948	0.044
s^2			0.0057
SE			0.029

In Table 4 the individual results for seven antilipoidal cerebrospinal fluids examined with CLC antigen are shown separately It is evident that the reactivity of CSF is influenced only to a very small extent by heat inactivation, the mean reduction in CLC titre following heat inactivation being comparable in magnitude only to that found in the

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If this is so, it may be concluded that in non-syphilitic sera there are immune globulins of uniform heat stability, in contrast to the heterogeneous immune globulin pattern in both early and late syphilis indicated by the discrepancy between CLC and CC titre reductions following heat inactivation

The reduction in antilipoidal titre following heat inactivation for non-syphilitic antilipoidal sera in this study (originating from Danish patients with a variety of non-syphilitic conditions, including one case of lepromatous leprosy) corresponds closely to the titre reduction demonstrated earlier in this laboratory for TPI non-reactive, antilipoidal sera from patients with lepromatous leprosy (originating from Madras, India) (6)

SUMMARY

Examination of the heat stability of the complement fixing antilipoidal immune globulins found in blood and CSF at various stages of the syphilitic infection and in non-syphilitic reactors was made on 191 sera and 7 CSFs

Significant differences as regards heat stability were demonstrated between antilipoidal antibody found in serum from cases of primary and secondary syphilis and in CSF from cases of tertiary syphilis on the one hand, and that found in serum from cases of tertiary and late latent syphilis on the other

Significant differences in heat stability were also demonstrated between the antilipoidal antibody in the above-mentioned syphilitic groups and the antilipoidal antibody found in serum from non-syphilitic reactors

The greatest heat stability was found in the CSF of tertiary syphilis and in serum from cases of early syphilis Late syphilis was characterized by decreased heat stability as compared with early syphilis and CSF, and the group of non-syphilitic reactors showed an even lesser heat stability

It is concluded that at least two forms of antilipoidal immune globulins with different heat stability are responsible for the antilipoidal complement fixing reactivity found in the sera and CSFs in this study

Comparison between the results obtained with ordinary lecithin containing cardiolipin antigen (CLC) and the lecithin-free cardiolipin antigen cardchol (CC) indicates differences between the immune globulins which react with these antigens, or differences in the sensitivity of these antigens to the various antilipoidal immune globulins involved The CC antigen is less sensitive to the more heat-stable immune globulins and more sensitive to the less heat-stable immune globulins than CLC antigen

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BRIEF REPORT

DEVELOPMENT OF THE GLOMERULUS AND JUXTAGLOMERULAR APPARATUS[1]

Preliminary report

By *Jerzy Kaźmierczal*

It is well known that in the new born pig nephronogenesis is not complete In the present study the kidneys of new born piglets were histologically examined in 1μ thick plastic sections stained with alkaline toluidine blue and in paraffin sections stained either with Mallory trichrome stain or with the method for metalophilic cells (*Marshall* (1)) Fresh frozen cryostat sections of these kidneys were used for histochemical demonstration of adenosine triphosphatase (ATPase) with Wachstein and Meisel s lead method (2) alkaline phospha
nique (3) glucose 6 phosphate dehydroge
technique (4) and with the method found
czal (5)) here called acid(glucose 6)phosphatase (A(g 6)Pase) Some animals were injected i v with colloidal carbon prior to laparathomy

Histological examination of the kidneys indicated that the narrow subcapsular layer of the renal cortex consisted of the vascularized mesenchyme differentiating into the primordia of the nephrons In the pocket like cleft of the differentiating nephron the presence of capillaries containing erythrocytes and in the injected animals particles of carbon has been frequently noticed (Fig 1) The examination of serial sections has confirmed the communication of these capillaries with the renal circulation though at first only with the small sinusoids which are numerous in the neogenic zone Then, the branches of the neighbouring arteriole merge into the sinusoidal capillaries buried in the hollow of the enlarging cleft (Fig 2) establishing the close arterio venous glomerular circulation observed to be present throughout all further stages of glomerulogenesis Next the lips of the cup formed from the cleft grow about and thus enclose the capillaries which originally formed the dilatated peripheral channels which in turn are progressively modulated by the visceral epithelium of Bowman s capsule

From the very early stages of glomerulogenesis a certain amount of free lying cells of mesenchymal appearance were present close to both the branches of the arteriole and the sinusoidal primary capillaries These cells could be differentiated from the endothelial cells by their unorganized position polymorphic appearance and by histochemical methods The latter have indicated that while the endothelial cells were selectively stained in the reaction for A(g 6)Pase (Fig 3) the mesenchymal cells both outside and in the developing glomerulus were strongly stained in the reaction for ATPase (Fig 4) Further observations of the developing glomerulus in histologically and histochemically treated sections indicated that the mesenchymal cells originally distributed in the vicinity of the capillaries of the differentiating glomerulus finaly established their position as the mesangial cells glomerular hilar (Goormaghtigh) cells and as the smooth muscle coat of afferent and efferent vessels The mesenchymal cells making up the mesangium and the two components of the juxtaglomerular apparatus have shown some features characteristic for reticular tissue Beside this morphological similarity some of these cells were capable of phagocytosing particles of carbon some of them were undergoing transformation to monocytes and granulocytes and also they were becoming metalophilic However the latter feature (as well as granules in the media of the vas afferent) was first prominent in the glomeruli which were relatively mature (*Fig 5*) On the basis of these observations it appears that all these components i e mesangium hilar (Goormaghtigh) cells the smooth muscle coat of the vas afferent

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1 Partly presented at the 8th International Congress of Anatomists in Wiesbaden 8 13 August 1965

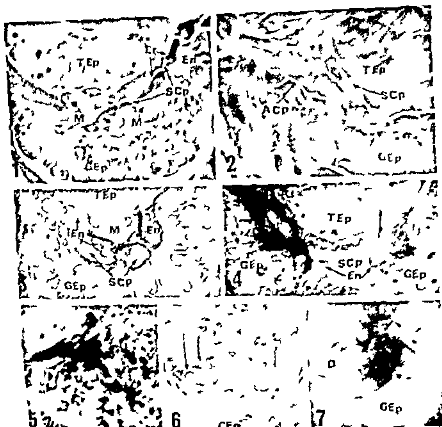


Fig 1 7

All ph tograj hs are taken fr m the ki lneys f tl e new l orn j igs
F gures 1 2 3 4 an l 7 demonstrate the S sl ap l stage of nej hr n g nesis

Fig 1 P cket l ke cl ft l mite l by the tul ular (TE$_{\mathrm{I}}$) an l glomerular (Cl ( ) ej l thelium S nuso dal cap llary (SCp w tl d tinct end thel al cells (En)

Fig

Fig 3 A

Fig 4

Fig 5 M ... s us dal capillary (SCp) are unstained 660 ×

Fig 6

Fig 7 Alkal ne phosphatase is d stinctly demonstrated in the pro pective j rox mal tubule wh le the epithel um of the pr spective renal corpuscle ((Ep) and of the d stal tubule (D) is unsta ned 300 ×

and probably efferent form a kind of glomerular and juxtaglomerular reticular system known to respond so well to various pathological stimuli

In the differentiating nephron the reaction for G 6 PDH has revealed some activity of this enzyme in the prospective tubular segment of the macula densa This reaction appeared in the more advanced stages to be present around the whole circumference of the growing tubule (Fig 6), similar to the reaction observed for AlkPase in the prospective tubular segment of proximal tubulus (Fig 7) However while the whole elongating proximal tubule during nephronogenesis was constantly alkaline ph s phatase positive the reaction for G 6 PDH became gradually restricted to the tubular cells in contact with the vas afferent and hilar cells of the mature glomerulus. Although the presence of the reaction for G 6 PDH in the prospective tubular seg ment of the macula densa cannot be taken as a proof of the functional abil ty of the cells it seems however to indicate the early preparation for function much earlier in fact than any other precursor cells of the juxtaglomerular apparatus which at this time are virtually primitive mesenchymal (reticular) cells probably not different from such cells elsewhere in the body

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SEXUAL DIFFERENTIATION IN THE XXXXY CHROMOSOME CONSTITUTION

Report of a Case in an 11 Year Old Boy

By

JØRGEN LAURITZEN, ANDERS FRØLAND and SVEND G. JOHNSEN

Received 25.II.65

Several different types of sex chromosome abnormalities are known in man. In the male they seem to be generally associated with pronounced hypogonadism. The most well known clinical picture is Klinefelter's syndrome with an XXY karyotype, but greater deviations from the normal chromosome complement are known, such as XXXY and XXXXY constitutions.

Mental deficiency is a common but not essential feature of Klinefelter's syndrome with XXY sex chromosomes. Patients having three or four Xes in addition to the Y are always mentally retarded. In addition to this XXXXY patients present a number of symptoms justifying the establishment of a special clinical entity.

We have recently studied a patient with many typical features of the XXXXY syndrome.

CASE REPORT

A boy aged 11½ years was referred to a surgical department for treatment of bilateral testicular ectopia.

He is an only child. At his birth the mother was 29 years old, the father 37 years old.

The pregnancy and delivery of the boy were uneventful. Weight at birth 2600 g, length 51 cm. It was noted that the ...

Our sincerest thanks are due to drs. *R. R. Race* and *Ruth Sanger*, London, for the determination of the Xg bloodgroups.

The technical assistance of Mrs. *Birgit Moestrup* is greatly appreciated.



Fig 1
The patient 11½ years old

There was pronounced bilateral epicanthus and some hypertelorism Ophthalmological examination showed alternating external strabism and a myopia of 20 diopters The background showed myopic degeneration Colour vision could not be tested The palate was higharched but no cleft was present There was no gynæcomastia the heart sounds were normal the spleen and liver not palpable

Th exter[illegible] 2) The penis was small [illegible] on the glans The scrotu[illegible] impressi n of labial folds [illegible] l placed at the external annuli They were small and soft

Examination of the upper extremities showed increased carrying angels with restricted pronation and supination movements The middel phalanx of the fifth finger on both hands was markedly shortened and radially deviating The feet showed valgus deformity, but no pes planus The fourth toe on both sides was [illegible]orly developed The spine was moderately scoliotic

X ray examination The scull was normal The upper extremities showed a proximal radio ulnar synostosis osseous on the right side fibrous on the left (Fig 3) The upp[illegible] The olecrana and processus conoidei were poorl[illegible] h fifth fingers was very short on both sides [illegible] valga Apart from this the lower



Fig 2

External genitals Note glandular hypospadia and bifid scrotum

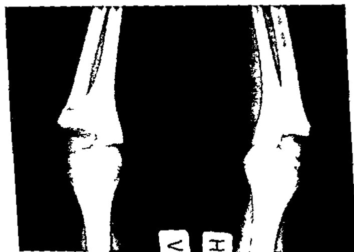
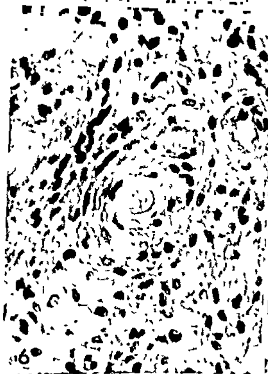


Fig 3

X ray pictures of the elbow joints showing bilateral radio ulnary synostosis







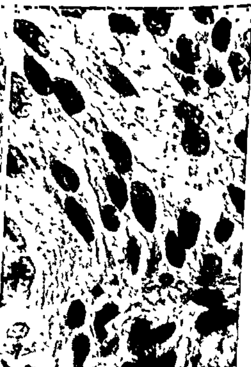


Fig 4 Right testis; × 35

Fig 5 Left testis; note at the bottom of Fig 4, the area with preserved tubules × 35

Fig 6 Right testis Structures resembling primordial follicles × 400

Fig 7. Left testis Area with Leydig cells Note the abundance of sex-chromatin bodies × 900



Fig 8
Nucleus from the buccal mucosa showing 3 Barr-bodies X 3000

extremities showed no abnormality Examination of the chest showed slight over all increase in the size of the heart Intravenous pyelography showed the right kidney to be rotated but no other anomaly

Hormone analysis Excretion of 17 ketosteroids 1 8 mg day Fractionation of 17 KS low function No significant testicular androgen production Urinary excretion of "total gonadotrophins 54MUU day (elevated)

Testicular biopsies (both testes) both biopsies involved a considerable part of the gonad Both gonads were very small—less than half the size normally seen in early childhood The small areas of gonadal tissue were surrounded by a thickened tunica albuginea (see Fig 4)

The greater part of both gonads consisted of hyalinized tubules In one of the gonads (left see Fig 5) a small area with non hyalinized tubules was seen These tubules had a mean diameter of 73 μ The epithelium contained only so called undifferentiated cells *i e* immature Sertoli cells Cells of the spermatogenetic line were completely absent

In both gonads a small number of unusual round or ovoid bodies was present (see Fig 6) These bodies were surrounded by a few concentric layers of cells with nuclei of unidentified type The center of the body was a structureless hyalinized mass Such bodies had in general some resemblance with primordial follicles of the ovary

In both gonads sparse areas with Leydig cells were seen Histologically these Leydig cells contained some granules in the cytoplasm and thus were histologically active Many Leydig cell nuclei showed 1 2 or 3 sex chromatin bodies (see Fig 7)

Bloodgroup studies The sex linked Xg^a bloodgroups were kindly determined by drs *R R Race* and *Ruth Sanger* The patient and his parents were all Xg (a+)

Cytological examination Sex chromatin was determined on Feulgen stained buccal smears 200 nuclei were scored 12 5 per cent contained three Barr bodies (Fig 7 Table 1)

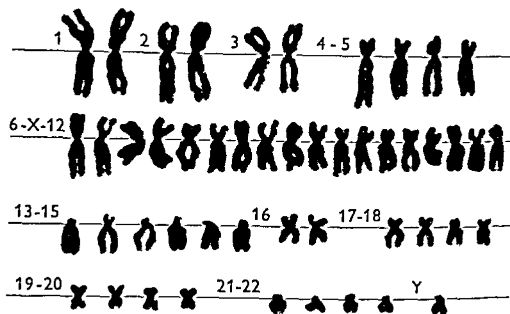


Fig 9
The karyotype of the patient 49 chromosomes with presumably 4 X chromosomes and a Y Skin culture Giemsa stain × 2400

TABLE 1
Sex Chromatin Studies
No of Barr bodies in the nucleus

	0	1	2	3	total
No of cells	20	73	82	25	200

The chromosomes were studied in cells from skin and blood cultures (*Froland* 1961, 1962) The distributions of the chromosome counts are seen in Table 2 The majority of the cells had 49 chromosomes The rest of the cells showed random losses of chromosomes

Analysis of cells with 49 chromosomes showed a pattern compatible with an XXXXY karyotype (Fig 9)

The sex chromatin of the parents was normal

TABLE 2
Chromosome Studies Count Distribution
No of chromosomes in the cell

	<46	46	47	48	49	>49	total
Blood	–	1		2	17		20
Skin	2	–	–	6	41	1	50
total	2	1		8	58	1	70

DISCUSSION

The XXXXY karyotype in an oligophrenic boy was first described in 1960 by *Fraccaro, Kaijser & Lindsten* (see also *Fraccaro & Lindsten* (1960)). Since then at least 40 cases have been reported. *Day et al.* (1963) have reviewed 9 cases. Oligophrenia and hypogenitalism are always present. Some of the following symptoms are very usual: Epicanthus, strabismus, myopia, malformed ears, cleft palate, radio-ulnar synostosis, short second phalanx of the fifth finger, coxa valga, pedes plani, cardiac malformation, small penis, underdeveloped scrotum and undescended testes. In Klinefelter's syndrome with an XXY or XXXY karyotype atrophy of the tubules of the testes is the only constant feature. The other symptoms are (apart from mental retardation) very uncommon and the external genitals are otherwise normal.

Thus the XXXXY abnormality seems to represent a characteristic clinical syndrome.

Of great interest for the understanding of the function of X and Y chromosomes in human genital development is the fact that patients with Klinefelter's syndrome (due to an XXY chromosome constitution) and patients with an XXXXY constitution display a great difference with regard to the development of their external genitals. As mentioned above, ordinary Klinefelter patients have normal penis, normal scrotum, normal epididymis etc. In the XXXXY syndrome the external genitals are poorly developed, and this underdevelopment originates in an abnormal sex differentiation in the foetus. (Our patient displayed hypospadia, bifid scrotum and retained testes.)

A male sexual differentiation in the human foetus is independent of the presence of germ cells. It is a wellknown fact that differentiation in male direction rests solely upon the Leydig cell function in the foetus (*Burnes* 1955). Thus it would appear that the major function of the Y chromosome is to cause a development of Leydig cells in the gonads which again leads to the production of steroids in the foetus capable of starting and completing the differentiation of the genital system into male direction.

It is clear that in the XXXXY syndrome the Leydig cells in the foetus must have had *some* function, otherwise the patient would have been a phenotypic female. It is therefore tempting to assume that the overwhelming amount of X chromosome material in the XXXXY syndrome can partly suppress the *influence of the* Y chromosome. Another possibility is that the Y chromosome is defective.

The presence of Leydig cells in the male foetal gonad can be traced back to th[illegible] *gonad itself* in early foetal [illegible]velopment of cortical and [illegible] the undifferentiated gonad. [illegible]

gonad structures which are similar to the formation of primordial follicles. Structures resembling primordial follicles in the XXXXY-syndrome were previously described by *Bunge & Bradbury* (1960), *Atkins et al.* (1963) and other authors. In the patient described by *Bunge & Bradbury* (1960) the structures were more similar to primordial follicles than in our patient, presumably because their patient was only five years old, while our patient was eleven years old at which age he already displayed signs of degeneration of the gonads.

By linking together abnormalities in the gonads (which presumably are due to *abnormalities in the cortical/medullary balance in early foetal life*) with abnormalities in the sex-differentiation of the external genitals, the XXXXY-syndrome gives a highly interesting contribution to the understanding of the function of the sex-chromosomes in man.

SUMMARY

In an oligophrenic boy aged 11½ years 49 chromosomes and an XXXXY karyotype were found. The most prominent clinical features were epicanthus, strabismus, myopia, radio-ulnar synostosis, short middle phalanx of both fifth fingers, hypospadia and a small bifid scrotum. Both testes were ectopic and small. The histological picture showed absence of germ cells, hyalinization of tubuli, Leydig cell hyperplasia and peculiar intratubular bodies resembling very primitive follicles. The urinary gonadotrophins were elevated.

The unusual feature of this syndrome gives important information on the mechanism of the sexual differentiation in man.

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ATHEROSCLEROSIS IN AN AUTOPSY SERIES

6 *Relation of Cerebral Atherosclerosis to Age and Sex*

By

J CHR GIERTSEN

Received 3 iv 65

Fatty streaks have been found in the cerebral arteries as early as in the second decade (*Baker, Iannone & Kinnard* (1)), but usually they appear later, either in the third (*Holman & Moossy* (9), *Wolkoff* (19)), or in the fourth decade (*Baker, Refsum & Dahl* (2), *Mathur, Patney & Kumar* (11)) Thus, as stressed by *Holman & Moossy* (9), visible atherosclerotic changes usually appear earliest in the aorta, namely already in the first decade, ten years later in the coronary arteries and about twenty years later in the cerebral arteries Subsequently, cerebral atherosclerosis advances progressively with age (*Baker, Iannone & Kinnard* (1), *Baker, Refsum & Dahl* (2), *Gore & Hirst Jr* (8), *Holman & Moossy* (9), *Mathur, Patney & Kumar* (11), *Meyer, Pepler, Meyer & Theron* (12), *Rabotti, Zibordi & Belgeri* (14), *Winter, Sayre, Millikan & Barker* (18), *Wolkoff* (19)) In the series of *Holman & Moossy* (9) and *Wolkoff* (19) all individuals were positive for cerebral atherosclerosis after the ages of 30 and 40 years, respectively On the other hand grossly normal cerebral arteries were found in 9 per cent of the individuals in the ninth decade by *Baker, Refsum & Dahl* (2), and in 16 per cent of the individuals in the seventh decade by *Mathur, Patney & Kumar* (11) In *Roberts Jr, Moses & Wilkins'* (15) series as many as 30 per cent of the individuals past 70 years of age displayed grossly normal cerebral arteries Thus, although cerebral atherosclerosis generally progresses with age, it is not an invariable concomitant of age, and even old individuals may have grossly normal cerebral arteries

It has previously been pointed out that the sex trend in aortic and coronary atherosclerosis is not quite clear (*Giertsen* (6, 7)) Most reports on cerebral atherosclerosis do not reveal any sex difference (*Baker, Iannone & Kinnard* (1), *Baker, Refsum & Dahl* (2), *Meyer, Pepler, Meyer & Theron* (12) [illegible]
Wi [illegible]
less [illegible]

and in *Rabotti, Zibordi & Belgeri*'s (14) series males had more cerebral atherosclerosis than females in the fifth, sixth, and ninth decades, whereas females prevailed in the seventh and eighth decades Thus the information on the sex trend in cerebral atherosclerosis is somewhat contradictory

MATERIAL

The material has been previously described (*Giertsen* (4 5)) It consists of 408 cases, 211 males and 197 females, ranging in age from 15 to 89 years The mean age was 62 3 years in males and 62 9 in females

In this paper the relation of cerebral atherosclerosis to age and sex will be considered The total colesterol phospholipoid ratio in the arterial wall—the ChPh value—has been used as index for the severity of atherosclerosis (*Giertsen* (4))

The mean ChPh value has been calculated for each age and sex group and the mean age for each sex and ChPh group Furthermore, the correlation between age and ChPh value has been examined in each sex and in the total series Finally the significance of age and sex for the inter relationship between atherosclerosis in the aorta the coronary and the cerebral arteries has been examined

For all individual groups of 6 cases or more the standard error of the mean has been calculated

RESULTS

The Total and Mean Amounts of Atherosclerosis in the Total Series

The total amount of cerebral atherosclerosis (the sum total of all ChPh-values) was 444 72 (males 232 04, females 212 68) The mean in the total series (all ages combined) was 1 09, 1 10 in males, and 1 08 in females Thus, in this series, in which the mean age is identical in the two sexes, there the mean amount of atherosclerosis is also identical

The Mean Amount of Atherosclerosis in the Age-Sex groups

Table 1 and Fig 1 show that the mean ChPh-value increases almost straight-lined with age The rise is most steep in the last three decades

TABLE 1

Cerebral Arteries The Mean ChPh Value and the Standard Error of the Mean within each Age Group in the two Sexes and in the Total Series

Age group	ChPh value					
	Males		Females		Total	
	Mean	S E	Mean	S E	Mean	S E
15–19	0 43				0 43	
20 29	0 51		0 46		0 48	0 06
30–39	0 63	0 12	0 64	0 10	0 63	0 08
40–49	0 89	0 15	0 51	0 06	0 71	0 09
50 59	0 94	0 12	0 78	0 11	0 86	0 08
60–69	1 06	0 10	1 04	0 10	1 05	0 07
70 79	1 42	0 11	1 41	0 11	1 42	0 08
80 89	1 45	0 11	1 68	0 16	1 57	0 10
All age groups	1 10	0 05	1 08	0 05	1 09	0 04

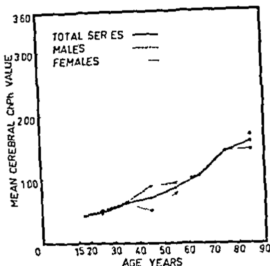


Fig 1

Cereb al atheroscleros s The variation of the mean ChPh value within the age groups in the total series and in each sex

In the first two age groups where both sexes are rej resented the period 20 to 39 years the mean values are practically identical In the next two age groups the male value is the highest Then the values are once more practically identical in two decades and in the last decade the female value is the highest The sex difference is significant only in the 40–49 year age group Thus males seem to have more cerebral atherosclerosis than females in middle life and the value for males in the fourt es is reached by females some 15 years later

The Mean Age at Specified Amounts of Atherosclerosis

Table 2 and Fig 2 show that the mean age increases almost linearly with the ChPh value to the 1 20 1 39 ChPh group Subsequently there are only minor variations with a further increase of the ChPh value

Furthermore in the first ChPh group where both sexes are represented males show a mean age almost 9 years less than females However with increasing ChPh value there is no definite sex trend except in the interval 1 60 2 79 where the mean age is slightly lower in males than in females but not significantly so

The Correlation between Age and Atherosclerosis

Table 3 shows the correlation between age and atherosclerosis in each sex and in the total series In the latter the correlation coefficient was +0 43 in males +0 37 and in females +0 48 The difference be

tween the z-transformed coefficients is not significant (*Snedecor* (17)) Thus, there is a fairly strong correlation between age and cerebral atherosclerosis, but there is no sex difference in this correlation

TABLE 2

Cerebral Arteries The Number of Cases the Mean Age and the Standard Error of the Mean, within Specified ChPh Groups in the two Sexes and in the Total Series

ChPh-group	Age: Males No of cases	Males Mean	Males S E	Females No of cases	Females Mean	Females S E	Total No of cases	Total Mean	Total S E
0.00–0.19				7	41.1	2.6	7	41.1	2.6
0.20–0.39	19	45.5	3.2	19	54.1	2.8	38	49.8	2.2
0.40–0.59	52	53.8	1.9	41	55.7	2.7	93	54.6	1.6
0.60–0.79	26	60.6	3.1	33	56.1	2.5	59	58.1	2.0
0.80–0.99	22	66.6	3.6	16	64.4	3.3	38	65.7	2.4
1.00–1.19	13	67.8	3.0	15	17.1	2.1	28	69.5	1.8
1.20–1.39	20	72.0	2.3	8	66.9	5.2	28	70.5	2.2
1.40–1.59	11	69.1	3.5	9	60.6	4.0	20	65.3	2.8
1.60–1.79	6	68.8	6.9	12	71.0	4.1	18	70.3	3.5
1.80–1.99	13	67.2	3.4	8	75.1	2.1	21	70.2	2.4
2.00–2.19	11	70.8	3.6	4	76.3		15	72.3	2.9
2.20–2.39	3	68.3		12	74.0	2.2	15	72.9	2.6
2.40–2.59	6	63.7	6.6	4	72.8		10	67.3	4.2
2.60–2.79	3	73.0		4	76.0		7	74.7	3.2
2.80–2.99	3	72.7		2	66.0		5	70.0	
3.00–3.19	2	60.5		2	75.5		4	68.0	
3.20–3.39									
3.40–3.59	1	73.0		1	80.0		2	76.5	

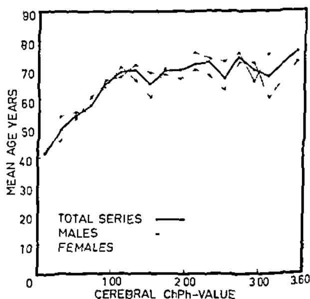


Fig 2

Cerebral atherosclerosis The variation of the mean age in the ChPh groups in the total series and in each sex

TABLE 3

Cerebral Arteries The Correlation between the ChPh Value and Age in the two Sexes and in the Total Series

Age	0 00–0 19	0 20–0 39	0 40–0 59	0 60–0 79	0 80–0 99	1 00–1 19	1 20–1 39	1 40–1 59	1 60–1 79	1 80–1 99	2 00–2 19	2 20–2 39	2 40–2 59	2 60–2 79	2 80–2 99	3 00–3 19	3 20–3 39	3 40–3 59
									Males									
15–19		1	1	1														
20–29		2	1															
30–39		4	8	3	1								1					
40–49		4	6	2	1	1		1	1	1	1	1						
50–59		4	17	3	1	1	2	1		5	1		1			1		
60–69		4	13	9	6	6	7	4	2	1			2	1	1	1		
70–79			5	7	8	2	7	2	1	4	6	1	2	1	2			1
80–89			1	1	4	3	4	3	2	2	3	1		1				
							Correlation coefficient r = +0.37											
									Females									
15–19																		
20–29			5															
30–39	3	3	3	7			1	1	1									
40–49	3	4	4	3	3													
50–59	1	5	8	8	2	1	1	2	2						1			
60–69		4	14	5	4	7	2	4		1	1	3	1	2				
70–79		3	5	9	5	4	2	2	5	5	1	7	2		1	2		
80–89			2	1	2	3	2		4	2	2	2	1	2				1
							Correlation coefficient r = +0 48											
									Total series									
15–19		1	1	1														
20–29		2	6		1													
30–39	3	7	11	10	1		1	1	1				1					
40–49	3	8	10	5	4	1		1	1	1	1	1						
50–59	1	9	25	11	3	2	3	3	2	5	1		1		1	1		
60–69		8	27	14	10	13	9	8	2	2	1	3	3	3	1	1		
70–79		3	10	16	13	6	9	4	6	9	7	8	4	1	3	2		1
80–89			3	2	6	6	6	3	6	4	5	3	1	3				1
							Correlation coefficient r = +0 43											

The Significance of Age and Sex for the Inter Relationship between Atherosclerosis in Different Arteries

Table 4 shows for each ChPh group of 15 cases or more in the cerebral arteries the correlation between the corresponding ChPh values and age in the aorta and in the coronary arteries It appears that although some coefficients are very small, or even negative, most of the coefficients are positive and fairly high No subdivision into sex groups has been made as most groups would be numerically small

However Table 5 and Fig 3 show the mean aortic and coronary ChPh values which correspond to specified cerebral values in each sex In some groups there is a slight difference between the sexes, but no sex prevails systematically over the other

TABLE 4

The Correlation between Age and the Aortic and Coronary ChPh Values which Correspond to Specific Cerebral Values

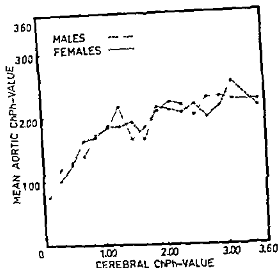
Cerebral arteries ChPh value	No of cases	Correlation ChPh value age: Aorta r	Correlation ChPh value age: Coronary arteries r
0 20–0 39	38	+0 80	+0 66
0 40–0 59	93	+0 63	+0 41
0 60 0 79	59	+0 74	+0 56
0 80 0 99	38	+0 68	+0 49
1 00 1 19	28	+0 16	—0 11
1 20–1 39	28	+0 63	+0 48
1 40–1 59	20	+0 10	—0 56
1 60 1 79	18	+0 59	+0 08
1 80–1 99	21	—0 02	—0 24
2 00–2 19	15	+0 47	+0 05
2 20–2 39	15	+0 08	+0 48

TABLE 5

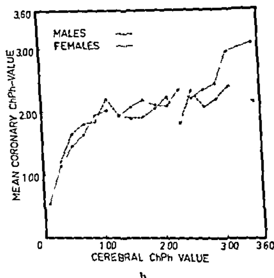
The Cerebral ChPh Value Compared with the Corresponding Mean Aortic and Coronary Values in each Sex (The Case Distribution is Given in Table 2)

Cerebral arteries	ChPh-value: Aorta, Males Mean	Aorta, Males S E	Aorta, Females Mean	Aorta, Females S E	Coronary arteries, Males Mean	Coronary arteries, Males S E	Coronary arteries, Females Mean	Coronary arteries, Females S E
0 00–0 19			0 76	0 06			0 54	
0 20 0 39	1 01	0 10	1 18	0 06	1 20	0 13	1 14	0 13
0 40–0 59	1 27	0 07	1 30	0 07	1 65	0 09	1 45	0 09
0 60–0 79	1 64	0 10	1 39	0 09	1 81	0 10	1 63	0 11
0 80–0 99	1 68	0 10	1 73	0 12	1 84	0 10	1 95	0 13
1 00–1 19	1 86	0 17	1 83	0 12	2 21	0 11	2 02	0 06
1 20–1 39	1 85	0 10	2 17	0 22	1 95	0 09	1 93	0 26
1 40 1 59	1 92	0 19	1 64	0 10	1 91	0 10	2 08	0 11
1 60–1 79	1 65	0 15	1 83	0 09	1 92	0 02	2 19	0 07
1 80 1 99	2 15	0 10	2 07	0 12	2 05	0 11	2 11	0 12
2 00–2 19	2 10	0 09	2 22		2 24	0 14	2 09	
2 20–2 39	2 05		2 18	0 11	1 82		2 35	0 09
2 40 2 59	2 18	0 18	2 00		2 33	0 29	2 21	
2 60–2 79	1 97		2 28		2 07		2 34	
2 80–2 99	2 14		2 29		2 19		2 44	
3 00–3 19	2 53		2 24		2 41		2 95	
3 20–3 39								
3 40–3 59	2 12		2 22		2 16		3 08	

The correlation between the corresponding ChPh-values in the aorta and the cerebral arteries, and in the coronary and the cerebral arteries have been previously examined (*Giertsen* (6, 7)) The coefficients for the former correlation were +0 60 and +0 69, and for the latter +0 40 and +0 59 Only the difference between the two latter coefficients is significant



a



b

Fig 3 a and b
Cerebral atherosclerosis The variation of the mean aortic and coronary ChPh values which correspond to specified cerebral values in each sex

DISCUSSION

The findings confirm that the degree of cerebral atherosclerosis, on an average, increases with age However, there are wide individual variations within the age groups indicating that old age is not invariably attended with severe cerebral atherosclerosis The correlation be-

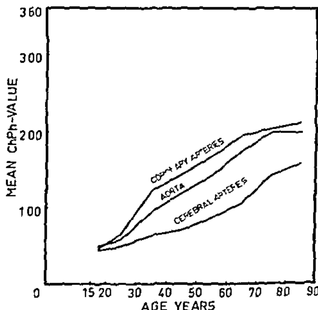


Fig 4

Comparison between atherosclerosis in the aorta the coronary and the cerebral arteries The variation of the mean ChPh value of the three arteries within the age groups in the total series

tween age and atherosclerosis is somewhat weaker in the cerebral arteries than in the aorta and the coronary arteries, where the correlations coefficients were + 0 66 and + 0 52, respectively (*Giertsen* (6, 7)) Both in the aorta and the coronary arteries the correlation between age and atherosclerosis was stronger in females than in males In the cerebral arteries the coefficient was also highest in females, but the sex difference was not significant

As mentioned in the introduction, visible changes appear at a different age in the aorta, the coronary, and the cerebral arteries This might suggest that the process starts at a different age in these arteries However, microscopical examinations indicate that coronary atherosclerosis, at least, starts simultaneously with aortic atherosclerosis, namely already in the first decade (*Fangman & Hellwig* (3), *Lober* (10), *Moon & Rinehart* (13)) Similar examinations of the cerebral arteries have not been found Nevertheless it might be that the atherosclerotic process starts simultaneously in all the three arteries, although the visible changes appear at a different age

This seems to be supported by the fact that the ChPh values are practically identical in the three arteries in the 15 19 year age group, viz 0 43 in the cerebral arteries, compared to 0 48 in the aorta, and 0 45 in the coronary arteries (*Giertsen* (6, 7)) Admittedly, the number of cases is small, but the finding suggests that the three arteries have reached, chemically, the same stage of atherosclerosis at this age

In all the subsequent age-groups atherosclerosis is, on an average most severe in the coronary arteries, less severe in the aorta, and least

severe in the cerebral arteries (Fig 4). This is not in agreement with the results in the majority of earlier investigations The discrepancy may possibly be due to the different grading methods used, a problem which has been discussed in previous papers (*Giertsen* (4, 5))

The cerebral arteries may or may not display visible changes within the ChPh interval 0 27–1 24 (*Giertsen* (4)) The range of this interval is somewhat greater than in the coronary arteries (0 37–1 00), and considerably greater than in the aorta (0.38 0 51) This indicates that the cerebral arteries can tolerate a higher rise in the ChPh-value than any of the two other arteries and still appear normal

It appears from Table 1 that not until the eighth decade is the mean ChPh value higher than the interval mentioned This means that visible changes, on an average, cannot be expected with certainty in the cerebral arteries until after 70 years of age, both in males and females This is considerably later than in the two other arteries, namely 40 and 30 years later than in the coronary arteries for males and females, and 50 years later than in the aorta for both sexes However, again the great individual variations must be borne in mind Table 3 shows that all age-groups contain individuals with a ChPh-value within the interval, that is individuals which may or may not display visible changes In fact, in this series grossly normal cerebral arteries were found in all age-groups, even in the seventies (11 out of 103), and in the eighties (3 out of 49)

Fig 1 seems to indicate that females lag about 15 years behind males in the development of cerebral atherosclerosis in middle life However, the sex difference was significant in the fifth decade only Furthermore, it is unlikely that females should have less atherosclerosis in the fifth than in the fourth decade Thus, at least the female curve does probably not reflect the true course of atherosclerosis Therefore, the sex difference found is possibly not the true one, but in some way a product of the selection of the series Similar suggestions have been made with regard to the sex difference in aortic and coronary atherosclerosis in this series (*Giertsen* (6, 7))

It was found that the aortic and coronary ChPh-values may be related to age within specified cerebral ChPh-groups This indicates that, when the cerebral arteries are taken as basis for a comparison, the great individual variation in the inter relationship between atherosclerosis in the three arteries (*Giertsen* (5)) may in part be the result of the influence of the age of the individual Sex seems to be of no significance with regard to the mean aortic an[illegible]

SUMMARY

The relation of cerebral atherosclerosis to age and sex has been examined in 408 individuals, 211 males and 197 females, ranging in age from 15 to 89 years, and with a mean age of 62.3 in males and 62.9 in females. The total cholesterol/phospholipoid ratio in the arterial wall—the ChPh-value—has been used as index for the degree of atherosclerosis.

Cerebral atherosclerosis was, on an average, found to rise progressively with age, and the correlation between age and atherosclerosis was fairly strong. However, there were wide individual variations.

On an average, visible changes do not appear with certainty in the cerebral arteries until after 70 years of age. Grossly normal arteries may be found in the highest age-groups.

Atherosclerosis has possibly reached the same stage, chemically, in the aorta, the coronary, and the cerebral arteries in the 15–19 year age-group. In all subsequent age-groups coronary atherosclerosis is, on an average, most severe, aortic atherosclerosis less severe, and cerebral atherosclerosis least severe.

Females lagged about 15 years behind males in the development of cerebral atherosclerosis in middle life, but in the ninth decade they had slightly more atherosclerosis than males. The sex difference was significant only in the fifth decade.

At specified amounts of cerebral atherosclerosis there was no definite, consistent difference in age between the two sexes, except a lower age in males in the lowermost ChPh-groups.

The mean amounts of aortic and coronary atherosclerosis which corresponded to specified cerebral amounts increased with age. There was no sex difference in the correlation between cerebral and aortic atherosclerosis, but the correlation between cerebral and coronary atherosclerosis was slightly stronger in females than in males.

It is suggested that the selection of the series may have influenced the sex trend.

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GLYCOGEN STORAGE DISEASE

A Case of Generalized Glycogenosis Type 2 (Pompe's Disease)

By

TROND KLEGI

Received 5 iv 65

While glycogen storage disease (GSD) formerly was believed to represent a single disease entity, recent research has revealed that several types exist, differing according to the type of enzymatic defect

The affection of carbohydrate metabolism in GSD can occur as follows

A Deficiency of enzymes responsible for the normal breakdown of glycogen

B Formation of abnormal glycogen molecules, unfit for breakdown by ordinary enzymatic activity

C An influence actively driving the liver towards glycogen synthesis (8)

The following is a case report of GSD, type 2 (Pompe's disease) with main affections of the heart, liver, brain and kidney

CASE REPORT

The patient was a girl who had 3 normal siblings The pregnancy and delivery were normal During the first 4 months nothing abnormal was detected except peculiar, involuntary movements of the extremities

At the age of 6 months she caught a highly resistant cough and her appetite diminished During the next 14 days the respiration became increasingly stressed and she was admitted on suspicion of a pneumonia

On admission she seemed seriously ill with forced respiration decreased pulmonary sounds on the left side but without cyanosis X ray revealed a marked density ... rmal of the left pulmonary area suggesting ar ... o

These findings gave rise to the suspisio ... ac scopy was performed However as the ... ed arrest developed Intubation and resuscitation was started immediately co ... with i m and intracardial vasopressors but the heart did not respond to any stimuli

Autopsy (RHO 0 200 64)

The left thoracic cavity was occupied by an enormously enlarged heart, while the lung was completely atelectatic The heart weighed 170 g (normal 40 g) and showed a marked thickening of both ventricles (Fig 1)

There was a similar, considerable liver enlargement, 335 g (normal 190 g) The remaining organs were macroscopically normal



Fig 1
The affected heart weighing 170 g (left) compared with heart of normal size
Note marked thickening of ventricular wall

Microscopic examination revealed a myocardium of a peculiar ' lace-work consistence, with broad, clear central spaces surrounded by a thin band of muscular fibrils (Figs 2 and 3) Special staining demonstrated heavy glycogen deposits in the periphery of the muscular fibre cells leaving a large, clear space in the centres (Fig 4)

The liver parenchyma showed a moderate vacuolization and increased storage of glycogen (Figs 5, 6 and 7) There was no marked fibrous tissue proliferation

Special staining for lipids was negative in both organs

Examination of different brain areas revealed increased, mesh-like cytoplasm with glycogen positive substance The findings were most outstanding in the dentate nuclei, the pons, globus pallidus, inferior olive and cerebral nerve nuclei The observations were consistent with a cerebral glycogenosis

Scarce glycogen deposits were observed also in the loop of Henle and in the distal convoluted tubules

DISCUSSION

GSD is considered to involve "inborn errors of metabolism', which in most instances, seem to be inherited as Mendelian recessives (1, 6)

In spite of recent advances in the field of enzymatic diseases, it seems

Figs 2 3

Fig 2 Myocardium cross section Typical lace work *arrangement* with broad clear central spaces (Haematoxylin eosine × 250)

Fig 3 Myocardium, longitudinal section Partial preservation of muscular fibrils demonstrating transverse and longitudinal striation (PTAH × 1000)



Figs 4–5

Fig 4 Glycogen deposits in myocardium with central spaces due to mechanical splitting of fibres Best's carmine × 1000)

Fig 5 Vacuolization of liver parenchyma No increase of connective tissue (Haematoxyline-eosine × 400)

Figs 6 7

Increased storage of glycogen in liver apparent as numerous intracellular granula of variable size (Best's carmine × 1000)

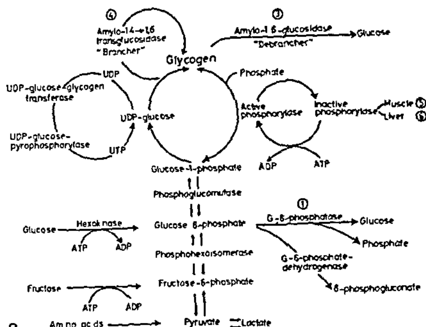


Fig 8

Diagram of formation and breakdown of glycogen The different enzymatic defects of the metabolic system are marked by encircled numbers from 1 to 6

natural to cite *Sokal et al* (8) who stated that "our ignorance far exceeds our understanding" with regard to the GSD

A survey is given in Fig 8, demonstrating the different enzymatic defects of the metabolic system according to *Andersen* (1) and *Cori* (3)

Type 1 (Von Gierke's disease) More than 100 cases have been reported, most authors claiming a glucose 6-phosphatase-deficiency Others doubt the reliability of these observations (8) The stored glycogen seems to be of normal type, mainly accumulating in the liver (1, 5, 6) Some cases are probably not diagnosed, others may be mistaken for lipoid storage disease (1) Very slight affections among close relatives have been described (1, 6, 7)

Type 2 (Pompe's disease, "glycogenosis cardiac syndrome", "congenital rhabdomyoma of the heart") The first case was reported in 1932, but the aetiology still remains obscure (vide infra) The heredity is probably that of a Mendelian recessive, single, autosomal gene (6)

Usually the affection is general, but some authors (5) separate a cardiac type and a neuromuscular type from the rest

Type 3 (Forbe's disease, "limit dextrinosis") The term is derived from the abnormally short outer "branches" of the glycogen molecule The condition is ascribed to a deficiency in amylo 1,6 glucosidase ("debrancher") (1, 5)

Type 4 (Andersen's disease, "amylopectinosis") A formation of ab-

6

7

Figs 6 7
Increased storage of glycogen in liver, apparent as numerous intracellular granula of variable size (Best's carmine × 1000).

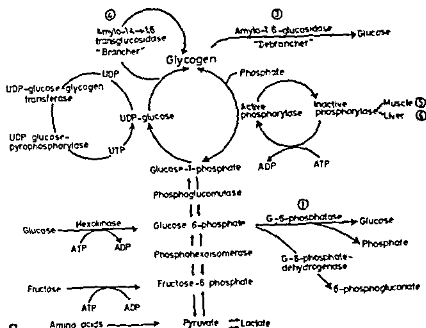


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Increased storage of glycogen in liver, apparent as numerous intracellular granula of variable size (Best's carmine × 1000)

of GSD type 2 is not considered very useful, as the glycogen storage may occur in almost every organ Hence, it has been observed in all divisions and layers of the alimentary tract, in the suprarenal glands, Henle's loop of the kidney, the pancreas and the liver, without fibrous tissue formation (5) Also, the mucous glands of the respiratory tract, the reticulo-endothelial tissue, thymus, lymph nodes and bone marrow sometimes show definite deposits of glycogen Even circulating leucocytes have shown increased content of glycogen

Our case differs from the usual picture in 2 aspects.

1 The liver was considerably enlarged, while hepato- and splenomegaly is considered very rare (5)
2 The cerebral affection was widespread and not confined to the cortical areas This fact may account for the patient's involuntary movements, suggesting an extrapyramidal origin

Differental diagnosis The many different types of congenital cardiac anomalies are always difficult to exclude The predisposition to pulmonary infections and mechanical obstruction brings different causes of pulmonary atelectasis into consideration; *e g* pneumonia, aspiration, bronchial foreign body

When the nervous system is affected, the picture may be confused with that of amyotonia congenita Some patients present a very large tongue and may be mistaken for cretins (2, 5, 8)

The microscopic picture can be confused with lipoid storage diseases, but these are excluded when special lipoid staining is performed

Because of the rare occurrence and variable clinical picture, the diagnosis is usually not made before autopsy However, a case in which the diagnosis had been established on the basis of muscle biopsy and glycogen structure analysis has been reported (5)

Therapy and prognosis The condition seems incompatible with normal life Most children die within few hours or days (1), probably from cardiac insufficiency due to mechanical splitting of the myocardial fibres by the stored glycogen

No causal therapy exists

SUMMARY

A report is given of a case of generalized glycogenosis, type 2 (Pompe's disease) The case was mistaken for bronchial obstruction with pulmonary atelectasis ,and cardiac arrest developed during bronchoscopy

Autopsy revealed marked cardio-hepato megaly, with a characteristic appearance revealed by microscopy The hepatomegaly in this type of glycogen storage disease is considered a rare occurrence

Signs of glycogen storage were detected also in the kidney and brain,

normally long outer "branches" in the molecule is considered the cause of the disease The deposits occur mainly in the liver and reticulo endothelial system, usually accompanied by fibrous tissue formation (1, 6)

Type 5 (McArdle-Schmid-Pearson's disease) The molecular structure of glycogen is similar to the one seen in type 3 The main disturbance is a deficiency in muscle phosphorylase, the glycogen content of striated muscle increasing to 4–5 times normal values (1)

Type 6 (Hers' disease) In many aspects the condition is obscure There is a lack of liver phosphorylase, with normal glycogen molecules The clinical picture is less severe than in type 1–5 Mode of heredity is probably a Mendelian dominant (1)

The present case clearly belongs to GSD type 2 (Pompe's disease), as the microscopic appearance and gycogen storage in heart muscle is considered pathognomonic The glycogenosis of liver (without fibrous tissue proliferation), brain and kidneys gives additional support to the diagnosis

Symptomatology The symptoms usually start at the age of 2-6 months, but the condition may be present at birth (4, 6) Often, the patients present a picture of growth failure, muscular weakness, anorexia, vomiting, drooling, tachycardia, dyspnoea and cyanosis They are prone to respiratory infections The enormous heart enlargement seen by X-ray is a most frequent observation Hepatomegaly, however, occurs rarely (5) When neurological symptoms supervene, the condition may be similar to amyotonia congenita (2)
Ecg-registration may show T-wave-alterations or depression of the ST-segment, but is seldom of diagnostic value (5)

Laboratory examinations reveal a normal blood sugar level, with normal glucose and galactose tolerance The response to glucagon and epinephrine injections is normal, and there is no acetonuria These observations are of value in the discrimination between this and other types of GSD

Biochemical investigations seem to conclude that the stored glycogen is of normal structure (7) Some authors consider the possibility of an aldolase deficiency (1), others a disturbance in hormonal regulation

Illingworth (7), stating that no enzymatic lesion has been uncovered, suggests a disturbance in the transmission of carbohydrates to lipids

Pathological-anatomical examination The typical "lacework"-appearance of the myocardium (see fig) does not appear in any other condition and is considered pathognomonic of the cardiac glycogenosis The glycogen content in fresh heart muscle may exceed 10 per cent of the total weight

Heavy deposits are often found in smooth and striated muscle, especially in tongue and diaphragm, combined with deposits in the central nervous system, preferably the pyramidal cells of the cerebral cortex (1) However, the division into a cardiac and a neuromuscular type

of GSD type 2 is not considered very useful, as the glycogen storage may occur in almost every organ Hence, it has been observed in all divisions and layers of the alimentary tract, in the suprarenal glands, Henle's loop of the kidney, the pancreas and the liver, without fibrous tissue formation (5) Also, the mucous glands of the respiratory tract, the reticulo endothelial tissue, thymus, lymph nodes and bone marrow sometimes show definite deposits of glycogen Even circulating leucocytes have shown increased content of glycogen

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Differential diagnosis The many different types of congenital cardiac anomalies are always difficult to exclude The predisposition to pulmonary infections and mechanical obstruction brings different causes of pulmonary atelectasis into consideration *e g* pneumonia, aspiration, bronchial foreign body

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Autopsy revealed marked cardio hepato megaly, with a characteristic appearance revealed by microscopy The hepatomegaly in this type of glycogen storage disease is considered a rare occurrence

Signs of glycogen storage were detected also in the kidney and brain

the localization of the cerebral glycogenosis being diffuse and widespread The finding is correlated to the involuntary, choreatic movements which were present from birth

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BODY WEIGHT AND TUMOUR GROWTH IN MICE

By

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Received 8 iv 65

In most strains of mice the body weight of the females is lower than that of males of similar age The problem therefore arises in experimental work as to whether it is correct to compare males and females of similar age, or males and females of similar weight

In the present study the situation is evaluated through the use of the Ehrlich ascites carcinoma as it is possible to assess both the host inflammatory response and its immune response to this tumour homograft (2)

MATERIAL AND METHODS

Mice of the closed colony kept at this Institute (1) and the Ehrlich a[illegible]
cin ma were used [illegible]
male mice The firs[illegible]
230 000 mmc The t[illegible]
dosage used was 0 1 [illegible] tumour ascites per mouse

Experimental Procedure

Intraperitoneal tumour growth Two groups of 15 male and 15 female mice were set up In one group the males and females were of similar age the mean weight (± S D)of the males being 29 2 ± 1 0 g and of the females 24.3 ± 1 0 g In the other group the mice were of equal weight the males and fem[illegible]

[illegible] were all killed 6 [illegible] tumour ascites was removed and measured The packed cell volume (PCV) of the cells in the tumour ascites was [illegible]

th[illegible]
ar[illegible]
w[illegible]

cc[illegible]

RESULTS

Intraperitoneal Tumour Growth

These results are shown in Table I

There was a statistically significant difference between the males in

the localization of the cerebral glycogenosis being diffuse and widespread The finding is correlated to the involuntary, choreatic movements which were present from birth

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TABLE 2

Subcutaneous Growth of Ehrlich s Ascites Carcinoma in Male and Female Mice of Similar Age and in Males and Females of Equal Weight
(15 Males and 15 Females in each Group)

Mice		Similar age	Equal weight
Mean tumour diameter (mm)	♂	30 5	39.8
	♀	23 6	25.2

4 males d ed due to technical error before close of experiment

DISCUSSION

In 1950 *Klein* (5) recommended the Ehrlich ascites carcinoma for use in the quantitative estimation of tumour growth He stressed that the survival time curves obtained following the intraperitoneal injection of comparable numbers of tumour cells were highly reproducible However there is a considerable scatter in the results for example he quotes a 50 *per cent mortality in* 14 days and 100 per cent mortality in 26 days Such a scatter can complicate the evaluation of differences in *different* experimental groups At the same time it should be remembered that death from this tumour is not dependent merely on tumour growth but also on the host s response to such growth (3) A closer analysis of the situation can be obtained from studies in which all the mice are killed on the same day as in the present experiment Using the *present methods tumour* growth the host s inflammatory response and its immune response can be assessed In addition such cross sectional studies from which fresh tumour ascites is available make cytological investigation possible—while survival time studies rule this out These possibilities of further investigation strengthen Klein s contention that the Ehrlich ascites carcinoma is an admirable tool in cancer research

To develop this theme further the present studies show that when male and female mice of similar age are used the total tumour volume produced 6 days after intraperitoneal injection of Ehrlich s ascites carcinoma is comparable On the other hand the inflammatory exudate produced *i e* the total fluid volume in the peritoneal cavity is not This at first sight suggests that there is a sex difference in inflammatory response But the male mice of similar age are larger than the females Thus it is reasonable to believe that the surface area available to respond to the inflammatory stimulus was greater in the males This is probably the case as if the total fluid volume is corrected for starting weight the fluid volume/g mouse is comparable Therefore there is no difference in host inflammatory response when mice of similar age are used and the total tumour volume in such mice is also *comparable*

However the difference in total fluid volume leads to a significant difference in the PCV of the cells in the *tumour* ascites in the two sexes This value is an expression of the nutritional conditions available to each individual tumour cell As the present experiment shows this dif

the two groups for all factors The younger males, *i e* those in the equal weight group, produced less tumour (0 01 > P > 0 001), less total fluid (0 001 > P), less fluid / g mouse (0 001 > P), and gave a higher PCV of cells in the tumour ascites (0 01 > P > 0 001) The females showed no significant differences, the respective P values being 0 6 > P > 0 5, 0 9 > P > 0 8, 0 8 > P > 0 7 and 0 3 > P > 0 2

TABLE 1

Intraperitoneal Growth of Ehrlich s Ascites Carcinoma in Male and Female Mice of Similar Age and in Male and Female Mice of Equal Weight (15 Males and 15 Females in each Group)

Mice		Similar age*	Equal weight
Mean total PCV of tumour cells (ml)	♂ ♀	1 59 1 74	1 16 1 49
Mean total fluid vol (ml)	♂ ♀	4 55 3 17	2 33 3 31
Mean total fluid vol./g mouse (ml)	♂ ♀	0 154 0 123	0 097 0 138
Mean PCV of cells in tumour ascites (%)	♂ ♀	22 4 35 4	33 3 30 6

* 1 male and 1 female mouse died before the close of the experiment

In the group of similar age the sex difference in mean total fluid volume was significant (0 001 > P), but the difference in fluid volume / g mouse was not (0 1 > P > 0 05) The total PCV of the tumour cells in this group showed no significant sex difference (0 4 > P > 0 3), but the PCV of the cells in the tumour ascites was greater in the females (0 001 > P)

In the group of equal weight, the PCV of the cells in the tumour ascites was similar in both sexes (0 6 > P > 0 5) All other factors showed significant sex differences, the males giving less total tumour (0 05 > P > 0 02), less total fluid (0 02 > P > 0 01) and less fluid / g mouse (0 02 > P > 0 01)

Subcutaneous Tumour Growth

The findings are shown in *Table 2*

The mean tumour diameter was greatest in the males in the equal weight group, being significantly greater than that in the males in the similar age group (0 02 > P > 0 01) The difference between the females was not significant (0 4 > P > 0 3) The diameter was greater in the males than in the females in both groups (0 001 > P)

TABLE 2

Subcutaneous Growth of Ehrlich's Ascites Carcinoma in Male and Female Mice of Similar Age and in Males and Females of Equal Weight (15 Males and 15 Females in each Group)

Mice		Similar age*	Equal weight
Mean tumour diameter (mm)	♂	30.5	39 8
	♀	23 6	25 2

* 4 males died due to technical error before close of experiment

DISCUSSION

In 1950 *Klein* (5) recommended the Ehrlich ascites carcinoma for use in the quantitative estimation of tumour growth He stressed that the survival time curves obtained following the intraperitoneal injection of comparable numbers of tumour cells were highly reproducible However, there is a considerable scatter in the results, for example he quotes a 50 per cent mortality in 14 days and 100 per cent mortality in 26 days Such a scatter can complicate the evaluation of differences in different experimental groups At the same time it should be remembered that death from this tumour is not dependent merely on tumour growth but also on the host s response to such growth (3) A closer analysis of the situation can be obtained from studies in which all the mice are killed on the same day, as in the present experiment Using the present methods tumour growth, the host s inflammatory response and its immune response can be assessed In addition such cross sectional studies from which fresh tumour ascites is available make cytological investigation possible—while survival time studies rule this out These possibilities of further investigation strengthen Klein's contention that the Ehrlich ascites carcinoma is an admirable tool in *cancer research*

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DISCUSSION

In 1950 *Klein* (5) recommended the Ehrlich ascites carcinoma for use in the quantitative estimation of tumour growth He stressed that the survival time curves obtained following the intraperitoneal injection of comparable numbers of tumour cells were highly reproducible However, there is a considerable scatter in the results, for example he quotes a 50 per cent mortality in 14 days and 100 per cent mortality in 26 days Such a scatter can complicate the evaluation of differences in different experimental groups At the same time it should be remembered that death from this tumour is not dependent merely on tumour growth, but also on the host s response to such growth (3) A closer analysis of the situation can be obtained from studies in which all the mice are killed on the same day, as in the present experiment Using the present methods tumour growth, the host s inflammatory response and its immune response can be assessed In addition such cross sectional studies from which fresh tumour ascites is available make cytological investigation possible—while survival time studies rule this out These possibilities of further investigation strengthen Klein's contention that the Ehrlich ascites carcinoma is an admirable tool in cancer research

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However the difference in total fluid volume leads to a significant difference in the PCV of the cells in the tumour ascites in the two sexes This value is an expression of the nutritional conditions available to each individual tumour cell As the present experiment shows this dif-

ference has not effected tumour growth under normal conditions, but such a difference might be of importance in, for example, studies on the effect of hormones on the action of antimetabolites The above findings suggest that it might be possible to obtain a similar PCV in both sexes by using males and females of equal weight, and this was found to be so If mice of similar weight are used, i e if the peritoneal surface available to respond is of similar area, a similar PCV of the cells in the tumour ascites can be obtained But this is obtained at the expense of a significant *difference in all the other factors, i e tumour growth and* both total fluid volume and fluid volume / g mouse That is to say neither tumour growth nor inflammatory response are then comparable

These findings suggested that to obtain comparable conditions of tumour growth it might be preferable to use mice of similar age after all—but to adjust the tumour dosage to starting weight This was done in a subsequent experiment (4) and it proved possible to obtain similar total tumour volume and PCV of the cells in the tumour ascites in both sexes in this way

The sex difference in subcutaneous tumour growth was marked, *tumour growth being greater in the males This difference* was increased when mice of equal weight were used As the males in this case were inevitably younger than the females, this is a demonstration of the *old* observation *(6)* that a tumour may grow *better in younger* mice At the same time, as subcutaneous tumour growth, in contrast to the usual intraperitoneal tumour growth, has been shown to be dependent of the host's immune response (2), it shows that the immune response of these younger males was less than that of the older males, which was in turn less that that of females of similar age Thus a weaker immune response in younger mice may explain the old observation

Two different transplants of the tumour were used in these experiments The tumour cell counts in these transplants were comparable and both were taken from 11-day transplants in male mice The female mice *in both groups in these experiments* are directly comparable—being of similar age and weight In these mice the results of tumour transplantation are similar in all respects There were no significant differences in *intraperitoneal tumour growth or inflammatory response* or in subcutaneous tumour growth These results stress the reproducibility and hence the degree of reliability of such observations

SUMMARY

Following transplantation of the Ehrlich ascites carcinoma *the* inflammatory response of male and female mice of similar age is comparable, while that of mice of equal weight is not The sex difference in the immune response to this tumour, that is obvious in mice of

similar age is accentuated using mice of similar weight. Comparable nutritional conditions for the tumour cells can be obtained in both sexes by using mice of similar weight or by adjusting tumour dosage to starting weight. There were no significant differences in the results for any of the factors investigated when two comparable tumour transplants were used.

These findings stress that in the Ehrlich ascites carcinoma we have a precision tool for use in cancer research.

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male mouse The tumour ascites was removed from the mouse One half was cen [illegible] The other half supplied the tumour

[illegible] placing the smallest possible drop [illegible] drawn Pasteur pipette The test [illegible] coarser pipette Then in [illegible]

[illegible] ere easily identified as such by their position Some tumour cells remained within his r ng of cells—but the rest were freely suspended in the fluid outside the r ginal drop

Experimental Procedure

The action of peritoneal fluid on tumour cells Wet preparations were made as described above us ng the peritoneal fluid from each of the 5 A Sn mice in turn and from each of the 5 closed colony mice

The action of mouse serum on tumour cells Wet preparations were made as above using whole serum from each of the mice and also both serum dilutions

Control slides were set up with saline and cell free tumour ascitic fluid in place of the test fluid All slides were exam ned for tumour cell lysis as described previously (7) after 30 and 60 minutes Readings were confined to tumour cells outside the original drop see above

RESULTS

Peritoneal fluid Table 1 shows that lysis occurred in all the peritoneal fluids from A/Sn mice and 4 out of 5 of the closed colony mice

TABLE 1

Tumour Cell Lysis in Peritoneal Fluid from 5 A/Sn Mice and 5 Closed Colony Mice Related to Time at 20° C

Test fluid	Mouse no	Degree of lysis	
		30 mins	60 mins
A/Sn per toneal fluid	1	0	+
	2	+	++
	3	+	++
	4	0	+
	5	+	++
Cl sed colony per toneal fluid	1	+	++
	2	0	++
	3	+	++
	4	+	++
	5	0	0
Saline		0	0
Ascitic fluid		0	0

++ complete lysis + some lysis 0 no lysis

Serum Table 2 shows that lysis failed to occur in whole serum With a 1 in 3 dilution lysis occurred with one A Sn and in 4 out of 5 closed colony sera The 1 in 6 dilution gave lysis in all cases With one A/Sn serum at both 30 and 60 minutes and with one closed colony serum at

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IN VITRO ONCOLYSIS AND ITS INHIBITION BY MOUSE SERUM

By

F Hartveit

Received 5 x 65

Recent work has indicated that homografted Ehrlich ascites carcinoma cells may become sensitised, *i e* antibody-coated, *in vivo* (7, 8) Such tumour cells may lyse on intraperitoneal injection into normal mice (1). It has been shown that lysis of such sensitised cells does not occur in the absence of complement (7) and is inhibited by tumour ascitic fluid (3) Thus the inhibitor may be a product of lytic tumour cells or a substance that is normally present in mouse serum, a substance that enters the peritoneal cavity in the inflammatory exudate called forth by the lytic tumour cells (9)

The Bergen A4 ascites carcinoma is, like the Ehrlich ascites carcinoma, a non-specific tumour It arose as a solid mammary carcinoma in an A/Sn mouse and has since been converted into the ascitic form (4) Cells from late transplants of this tumour have also been shown to be sensitised cells (5) Thus they too can be expected to lyse on intraperitoneal injection Furthermore it has been shown that the immunological lysis of such Bergen A4 cells is inhibited by tumour ascitic fluid (10)

The following *in vitro* tests were set up to test these two hypotheses firstly that cells of the Bergen A4 carcinoma should lyse when they come into contact with peritoneal fluid from normal mice, and secondly that the inhibitor(s) of immunological lysis may be a normal constituent of mouse serum As similar results have been obtained repeatedly only specimen experiments will be quoted here

MATERIAL AND METHODS

Peritoneal fluid and serum were collected from 5 adult male mice of strain A Sn and from 5 adult male mice of the closed colony kept at this Institute that is unrelated to strain A/Sn (10) The peritoneal fluid was centrifuged to obtain cell free fluid, which was stored overnight at —20° C The blood was obtained by bleeding from the heart and allowed to stand at 20° C until it clotted when it was centrifuged The supernatant serum was decanted at once and stored overnight at —20° C The following day all fluids were thawed quickly at 37° C and used at once The sera were used whole and also diluted 1 in 3 and 1 in 6 with physiological saline

Tumour cells from an eleven day transplant of the Bergen A4 ascites carcinoma that arose in a *mouse* of strain A/Sn were used The transplant had grown in a

if the serum is diluted with physiological saline Thus the inhibitor(s) of immunological lysis is a constituent of normal mouse serum and not a product of tumour cell lysis or a substance produced by the host in response to the tumour cells

The *in vitro* lysis in peritoneal fluid is in keeping with that seen *in vivo* with the Ehrlich ascites carcinoma (1), and with the finding that cells of the latter tumour lyse *in vitro* in media of low protein content if complement is present (7) The serum findings are in accordance with that mentioned in passing in an earlier paper (2) in which it was reported that cells of the Ehrlich ascites carcinoma did not lyse in whole mouse serum but did so if the serum was diluted 1 in 10 with physiological saline

The oncolysis described in these experiments can be compared to that which occurs when fresh human serum is added to ascitic tumour cells The latter has been thought to be due to the action of heterophil antibody in the human serum However it has recently been shown that the serum merely supplies complement to the system (8) In the present case where strain A/Sn mice were used any action of heterophil antibody in the test fluid is out of the question The finding that lysis may be more pronounced with the 1 in 3 than with the 1 in 6 serum dilution stresses that it is the balance between the amount of complement present and the amount of inhibitor that determines whether or not lysis occurs

Complement factor 1 is known to be present in the serum of normal mice of this closed colony (6) The fact that immunological lysis occurred when sensitised tumour cells were suspended in peritoneal fluid from these mice shows that the other complement factors were also present as such lysis is dependent on the presence of all four factors If complement is present in the peritoneal fluid, then it can also be expected to be present in the serum of the same mouse But lysis did not occur in serum Thus normal mouse serum must contain an inhibitor(s) of immunological lysis However when the serum, with its inhibitory factor was diluted with saline lysis occurred once more This confirms that complement was present in the serum

The same ... train A Sn peritoneal fluid and serum ... With closed colony fluids the lysis in ... homologous system—with A/Sn fluids the system is isologous Thus the latter results demonstrate lysis and inhibition of lysis of tumour cells sensitised in an otherwise genetically compatible system

The present experiments thus amount to an *in vitro* demonstration of the system of immunological tolerance to tumour tissue that has been shown to exist *in vivo* in the Ehrlich asc... nother ... sensi ... With ... cells and the isografted Bergen A4 cells

30 minutes and 2 at 60 minutes, lysis was more pronounced with the 1 in 3 than with the 1 in 6 dilution

Lysis failed to occur in the saline and ascitic fluid controls

TABLE 2

Tumour Cell Lysis in Mouse Serum Whole and Diluted 1 in 3 and 1 in 6 in Physiological Saline from 5 A/Sn and 5 Closed Colony Mice Related to Time at 20° C

Test fluid		Mouse no	Degree of lysis 30 mins	Degree of lysis 60 mins
A/Sn serum	whole	1	0	0
		2	0	0
		3	0	0
		4	0	0
		5	0	0
	1 in 3	1	0	0
		2	0	0
		3	0	0
		4	++	++
		5	0	0
	1 in 6	1	0	+
		2	+	++
		3	0	+
		4	+	+
		5	+	++
Closed colony serum	whole	1	0	0
		2	0	0
		3	0	0
		4	0	0
		5	0	0
	1 in 3	1	+	++
		2	+	++
		3	+	++
		4	0	0
		5	+	++
	1 in 6	1	+	++
		2	+	+
		3	0	++
		4	0	+
		5	+	+
Saline			0	0
Ascitic fluid			0	0

++ complete lysis + some lysis 0 no lysis

DISCUSSION

The present experiment demonstrates that sensitised tumour cells usually lyse in peritoneal fluid from normal mice, that lysis fails to occur in serum from the same animals and that lysis occurs once again

if the serum is diluted with physiological saline Thus the inhibitor(s) of immunological lysis is a constituent of normal mouse serum and not a product of tumour cell lysis or a substance produced by the host in response to the tumour cells

The *in vitro* lysis in peritoneal fluid is in keeping with that seen *in vivo* with the Ehrlich ascites carcinoma (1), and with the finding that cells of the latter tumour lyse *in vitro* in media of low protein content if complement is present (7) The serum findings are in accordance with that mentioned in passing in an earlier paper (2) in which it was reported that cells of the Ehrlich ascites carcinoma did not lyse in whole mouse serum but did so if the serum was diluted 1 in 10 with physiological saline

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The same argumentation applies to the strain A/Sn peritoneal fluid and serum But the conclusions are different With closed colony fluids the lysis and inhibition are occurring in a homologous system—with A/Sn [illegible] Thus the latter results demonstrate [illegible] tumour cells sensitised in an otherwise [illegible]

The present experiments thus amount to an *in vitro* demonstration of the system of immunological tolerance to tumour tissue that has been shown to exist *in vivo* in the Ehrlich ascites carcinoma [illegible] another [illegible] sensi- [illegible] With [illegible] Ehrlich cells and the isografted Bergen A4 cells

survive in spite of the host's immune response The present findings establish that this system is mediated via a normal constituent of mouse serum But they do not explain its mechanism which remains open to speculation

SUMMARY

Sensitised tumour cells of the non specific Bergen A4 ascites carcinoma that originated as a solid mammary carcinoma in a female A/Sn *mouse, were shown to undergo immunological lysis in vitro* in the peritoneal fluid of both isologous and homologous mice Serum from the same mice did not permit immunological lysis—but the property returned when the serum was diluted with physiological saline It is concluded *that a constituent of normal serum is responsible for this in*hibition of oncolysis caused by mouse complement that may lead to a form of immunological tolerance *in vivo*

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THE IMMEDIATE CAUSE OF DEATH IN MICE WITH EHRLICH'S ASCITES CARCINOMA

By

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Received 5 v 65

Death invariably follows the intraperitoneal injection of Ehrlich's ascites carcinoma cells in mice If a low dose of tumour cells is used solid tumour growth will result, but if a sufficiently large number of cells are injected an ascitic tumour develops (17) Although the underlying cause of death is clear the immediate cause of death has been shown to vary from mouse to mouse (7), some mice dying early from massive intraperitoneal haemorrhage, others dying later of pressure effects due to progressive tumour growth The mechanisms of the processes that bring about this variation were far from clear in 1961 However later work has provided further information on the mechanisms involved in ascitic tumour growth Enough evidence is now available to enable an analysis of the mode of death from ascitic tumour growth to be presented

MATERIAL AND METHODS

The following studies involve mice of the closed colony kept at this Institute (6 and the Ehrlich ascites carcinoma that has been kept by intraperitoneal transplantation in these mice for several years

The experimental material to be considered here has been used in three previous experiments In the first (6) referred to above 25 male and 25 female mice were each given an intraperitoneal injection of 0 1 ml of Ehrlich's ascites carcinoma The r [illegible]

were also given a similar injection of tumour ascites In these mice serial biopsy of the tumour ascites was carried out every third day Films were made and the morphology of the tumour cells studied

RESULTS

The first experiment showed that the mice of this closed colony fell into two distinct groups as regards their survival time following the intraperitoneal injection of Ehrlich's ascites carcinoma One group died relatively soon after transplantation, the other considerably later The

mice in the group dying early had more blood in the tumour ascites than those in the group with a longer survival time At the same time the amount of tumour ascites increased with the survival time in these mice

The second experiment showed that the number of injured tumour cells in the tumour ascites is directly related to the tumour blood content at six days

The third experiment showed that the early stage of tumour growth seen in the second experiment in which up to 30 per cent of the tumour cells are damaged is followed by a sudden transition to a stage in which few injured tumour cells are present This stage is in turn followed by the reappearence of injured tumour cells which increase in number until the mouse dies

DISCUSSION

Any analysis of the mode of death from intraperitoneal homotransplantation of the Ehrlich ascites carcinoma in heterozygous mice like those used in the present experiments must explain the bimodal distribution of the survival time shown in experiment 1 Shortly after that experiment it was suggested that the mice with a short survival time that died of massive intraperitoneal haemorrhage might be showing a Schwartzman like reaction to the genetically incompatible tumour (7) while those that survived longer died a mechanical death due to pressure effects caused by the progressively increasing tumour ascites It was suggested that the blood content of the tumour ascites in the former group could be regarded as an expression of resistance to Ehrlich s ascites carcinoma as genetical incompatibility between host and tumour was considered to be the underlying cause of the haemorrhage This idea was followed up As a result it was found that as shown in experiment 2 the tumour blood content in early transplants was directly related to the number of injured tumour cells found in the tumour ascites Now it has recently been shown that the volume of fluid exudate formed in the peritoneal cavity of mice with this tumour is also directly related to the number of injured tumour cells present and that the mere presence of uninjured tumour cells leads to fluid exudation (14) The latter finding is not unexpected as any non specific irritant may well call forth an inflammatory response The former finding that injured tumour cells may cause fluid exudation is also to be expected on the same grounds Indeed the release of autolytic enzymes from dead cells could be expected to give rise to a more severe inflammatory response than that to undamaged tumour cells Experiment 2 showed that this is so Extensive tumour cell damage is accompanied by haemorrhage Thus the inflammatory response that accompanies the presence of tumour cells in the peritoneal cavity will be accentuated when injured cells appear in the tumour ascites This in

flammatory response may be in excess of that causing maximal fluid exudation and haemorrhage may occur This haemorrhage may kill the mouse

But what is the cause of this tumour cell injury seen in these early transplants? It was previously felt that this injury might be due to the recipient s immune response to the tumour homograft and recent work has shown that this may on occasion be so but that it is not necessarily the case (14) In 1961 there was no reason to believe that the tumour cells injected could have carried antibody with them from their previous host Recent work has shown that this is possible—the cells injected may be antibody coated (12 and 13) Such sensitised cells undergo immunological lysis when placed in a medium of low protein content in the presence of complement (15)—*i e* on injection into a new host Previous sensitisation of the tumour cells is thus one of the causes of the tumour cell damage that leads to death from intraperitoneal haemorrhage In addition it is possible that the very fact that the tumour cells are antibody coated *i e* that they represent an antibody antigen complex may serve to increase the inflammatory reaction as it has been shown that such complexes may call forth such a response (19) Similarly it has been shown that C_1 esterase activated in the process of immunolog cal lysis may play its part in producing inflammation (21) On the other hand if unsensitised cells are injected such immunological cell damage will not occur at once The immune response of the recipient must first come into the picture and this may also lead to immunological cell damage Here we have the second cause of death from intraperitoneal haemorrhage In both cases the blood is "an expression of resistance to Ehrlich s ascites carcinoma (7)

However we know from experiment 1 that all the mice do not die from this cause Some survive to die later and these have less blood in their tumour ascites Experiment 3 gives us a clue to this problem as it showed that while many immunologically injured tumour cells are present in early tumour transplants this stage was followed by a sudden disappearance of such cells from the tumour ascites This disappearance of immunologically injured tumour cells coincides with the steady increase in the volume of the ascitic fluid in the peritoneal cavity that is *implied* in the progressive accumulation of tumour ascites demonstrated in experiment 1 This ascitic fluid has recently been shown to contain an inhibitor of *immunological tumour cell lysis* (11) *Thus the* inflammatory process brought about by immunological lysis is self limiting as *the inflammatory* response in itself leads to the formation of ascitic fluid that contains an inhibitor that prevents further lysis In other words *once this inhibitor* is present the tumour cells are protected from immunolog cal cell damage—a state of immunological tolerance has been established

This brings us to Holmberg s (16) observation that the tumour ascites and the serum of *tumour* bearing mice contain a substance that is toxic

to normal cells in tissue culture *Pall et al* (20), and *Burgess & Sylven* (2) have provided evidence that many of the mice with a similar ascitic tumour appear to be in a toxic state, about 6 days after transplantation from which some of them recover It is suggested that the mice in which intraperitoneal haemorrhage is *not fatal and those in which* haemorrhage has not occurred may still be suffering from the effects of Holmberg's toxic substance The finding that this effect is transient suggests that the substance is a product of cell lysis and that the supply fails when lysis ceases These substances are said to act primarily on the cell surface *It is thus possible that they could explain* the subcutaneous oedema (22) that occurs on the abdomen of the mice at this time This oedema subsides later (personal observation) This also suggests that its cause is related to tumour cell injury

As experiment 1 shows the amount of tumour ascites continues to increase in these mice that survive the effects of immunological lysis The increase in tumour volume in these mice is accompanied, as shown in experiment 3, by the reappearance of injured tumour cells The damage in these *cells differs* morphologically from immunological *damage* This differentiation is supported by the later finding that it occurs at a time when the tumour volume is excessive

However, the result of cell damage—irrespective of its cause—is the same The products of these dead cells once again lead to an inflammatory *response, a further increase in ascitic fluid and even to haemor-*rhage In contrast to immunological lysis there will be no reprieve from anoxic lysis as it occurs in the presence of the inhibitor of lysis

By this stage in the life history of the transplant little haemorrhage will be needed to kill the host as the mice are already moribund from *the effect of the progressive accumulation* of tumour ascites These effects will in part be mechanical due to pressure on the diaphragm and bowel as *Stuart & el Hassan* (22) have also suggested In addition the effects of electrolyte imbalance must be taken into consideration The massive ascites finally prevents the mice from moving freely, so they neither eat nor *drink In addition Holmberg's* (16) *toxic substance* may also serve to increase the general electrolyte imbalance

Further it has been shown that proteolytic enzymes are released into the ascitic *fluid from ascites tumour cells These enzymes* are said to come from uninjured tumour cells and it has been suggested that they are responsible for tumour invasiveness (see 1) But there is a snag here Are there any truly uninjured tumour cells present during the greater part of tumour growth? When immunologically injured tumour cells *disappear* from the tumour ascites—that is to say when the stage of immunological tolerance is reached —the morphology of the tumour cells *changes* They shrink, and may even become angular Intercellular bridges appear and the cells may agglutinate (10) *These* changes are compatible with life and with proliferation (9) But are these cells normal? The appearance of the morphological changes coincides with the

ability of the cells to lyse in the presence of complement (13) Thus these cells are probably immunologically sensitised cells—cells that are antibody-coated Is it not possible that this union of antibody and antigen could have changed the surface properties of the tumour cells—allowing the leakage of these proteolytic enzymes? If sensitisation is the cause of this leakage, and leakage a prerequisite for invasion, this could explain the malignant properties of these tumour cells

The morphological changes described above are strikingly similar to those described by *Dacie* (3, 4) in sensitised erythrocytes As he points out the latter changes have been shown to be associated with some loss of metabolic activity (5) There is a possible connection here with *Klein & Revesz's* (17) finding that tumour growth is not truly exponential in the later stages It is claimed (18) that this is not entirely due to failure of nutritional supplies, but to gradual reduction in the metabolic activity of the cells Consequently the time between consecutive mitosis becomes progressively longer Could it be that progressive sensitisation of the tumour cells is responsible for this progressive deterioration in reproductive ability?

The main findings discussed here and their coordination into an analysis of the mode of death from Ehrlich's ascites carcinoma can be summarized as follows Firstly the tumour cells injected will exert a non specific irritant effect in the peritoneal cavity The increase in capillary permiability in response to this will lead to fluid exudation and, as the amount of tumour increases progressively, so this exudation will increase and fluid will also accumulate in the peritoneal cavity In addition to this comes the inflammatory response to immunological lysis This may be great enough to allow bleeding, which may kill the mouse before the next stage of tumour growth is reached This stage—the protected stage—is accompanied by a reduction in the number of immunologically injured tumour cells due to the appearance of an inhibitor of immunological tumour cell lysis in the ascitic fluid Reduction in the number of injured cells will lead to a reduction in the response to such cells and so fluid formation will return to a lower level—that due to the irritant effect of the protected tumour cells

As heterozygous mice were used their response to inflammatory stimuli can be expected to differ So the mice who responded most will now be dead The survivors who escaped with or without a lesser degree of haemorrhage will now have reached a state of immunological tolerance due to the presence of inhibitor At this time anoxic lysis, which is not effected by the inhibitor, makes its appearance The products of such cell damage may also lead to fluid exudation or even bleeding Little bleeding will now be sufficient to kill the mice which at this stage are suffering from the pressure effects of their massive ascites and also from electrolyte imbalance On the other hand if the response to the inflammatory stimulus is not sufficient to give haemorrhage death from the latter two causes is inevitable

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SUMMARY

An analysis of the different modes of death of heterozygous mice following the intraperitoneal homotransplantation of Ehrlich s ascites carcinoma is given on the basis of differences in their response to the inflammatory stimuli provided by

1 *The tumour cells acting as a non specific irritant*
2 The products of lysis of tumour cells that have been presensitised by the immune response of the donor mouse
3 *The products of lysis of tumour cells actively sensitised by the* immune response of the recipient
4 The products of lysis of tumour cells suffering from anoxic injury

It is pointed out that the inhibitor of immunological lysis that appears in the ascitic fluid *i e* in the resultant inflammatory exudate has no inhibitory action on the lysis due to anoxic damage The consequences of anoxic damage are therefore irreversible, in contrast to immunological damage which is self limiting

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into two tubes The contents of one of the large tubes was divided into two equal portions To one of them was added Carbonyl Iron Powder S F (Fine Dyestuff and Chemical Ltd Manchester, main particle size 1 4 µ) in the proportions 1 ml plasma to 2 mg iron powder To the other portion was added a quantity of *Staphylococcus aureus* bacteria grown on horse blood agar made up at 120° and freshly suspended in broth not containing serum plasma or bovine serum albumin

The other large tube was centrifuged for five minutes at 800 r p m and the deposit washed three times with physiological saline as described by *Nordqvist et al* (5), enabling most of the neutrophil leucocytes to be collected without further centrifugation These cells were then suspended in an adequate volume of physiological saline This was divided into two portions to which were added iron powder and staphylococci as described above

All tubes were incubated for two hours at 37° and gently shaken every five minutes

RESULTS

Some twenty experiments were performed and they all showed the same tendency Within half an hour practically all the neutrophiles in plasma from the controls had phagocytized enough iron powder to acquire a completely black colour and lacked visible structures under the microscope After an equal length of time 50 to 75 per cent of the neutrophiles from the two patients had ingested iron powder and after another hour and a half all of them had done so

The control and patient neutrophiles suspended in physiological saline showed similar phagocytizing behaviour as the corresponding neutrophiles suspended in the individual's own plasma

Neutrophiles suspended in plasma from the two patients and controls had definitely phagocytized *Staphylococcus aureus* to approximately the same extent after incubation for one hour, unlike the washed neutrophiles suspended in physiological saline from any of the subjects

DISCUSSION

The consensus of opinion has been that an essential condition for efficient phagocytosis usually is the prior coating of the bacteria or other particles with an opsonin, either in the form of specific antibody or some less well defined serum protein, and subsequent combination with other serum factors Antigenically "inert" particles have been widely employed, among other things as controls in studies with bacteria and immune sera When it had been shown that at least two serum components—complement and specific antibody—were specifically required for phagocytosis of starch (6), it appeared fallacious to regard any particle as antigenically inert Furthermore, bentonite particles are only opsonized following direct adsorption of each of several preparations of gammaglobulin (7) But such coated bentonite particles are also phagocytized by washed blood cells that have been resuspended in Tyrode's buffer Although one cannot exclude the possibility that

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PHAGOCYTIZING CAPACITY OF THE NEUTROPHIL LEUCOCYTE IN PRIMARY "ACQUIRED" HYPOGAMMAGLOBULINAEMIA

By

OLOF FORSSMAN

Received 14 v 65

The literature on the phagocytizing mechanism has been dominated by the action of humoral factors But this mechanism can best be explained as a surface energy phenomenon in which cellular factors too are involved (1) With respect to ingestion of pneumococci by lung phagocytes and similar processes it has thus been demonstrated that even when there are no circulating antibodies the cellular defences may rid the body of the bacterial infection (2) This implies that the surface energy change required for phagocytosis results not mainly from intervention of specific immune substances but rather from forces acting in the phagocyte itself Accordingly the defence cells must be subject to local modifications If so, one would expect the neutrophiles to possess an intact phagocytizing capacity in hypogammaglobulinaemic conditions The aim of the present investigation is to illuminate this problem

MATERIALS

Two patients with "acquired" primary hypogammaglobulinaemia were examined on several occasions One of them was identical with Case 1 in a previous report (3) This patient was a woman of 43 belonging to blood group A in whom no anti B could be demonstrated by conventional means Immunoelectrophoresis disclosed no manifest immune globulins The anti human globulin inhibition test for immune globulins showed that the patient s serum continued to absorb antibodies against human immune globulin in a dilution of 1 20 but not in a dilution of 1 200 (normal serum loses its absorbtive power in dilutions between 1 30 000 and 1 60 000) No plasma cells were encountered in several bone marrow smears ASTA less than 2 units The other patient was a woman of 64 who had been examined in another hospital but meets the criteria for primary "acquired" hypogammaglobulinaemia[1]

Several healthy women were used as controls

METHOD

The procedure of *Bonnin et al* (4) was adopted for concentration of the leucocytes After centrifugation of 60 ml heparinized blood for ten minutes at 3000 r p m half

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[1] The author is indebted to Doctor O *Nordenfelt* MD of Jonkoping Hospital for his kind permission to publish this case

transfer this sensitivity to normal subjects by subcutaneous injection of hypogammaglobulinaemic leucocytes but not of their sera (22)

SUMMARY

Neutrophil leucocytes from two patients with primary "acquired" hypogammaglobulinaemia and from several controls were compared with respect to their capacity for phagocytizing iron powder and *Staphylococcus aureus* The following observations were made

1 Maximal phagocytosis of iron powder took place in both groups, although rather more slowly by neutrophiles from the patients
2 Plasma factors were not required for this process because washed neutrophiles suspended in physiological saline possessed the same phagocytizing capacity
3 Engulfment of *Staphylococcus aureus* took place with neutrophiles from both patients and controls when the cells were suspended in their own plasma but not when they had been washed and were suspended in physiological saline

Accordingly the cellular defence mechanism in the form of the phagocytizing capacity *in vitro* of the neutrophiles remained intact in the patients despite the fact that their level of opsonins particularly antibodies to *Staphylococcus aureus* must have been excessively reduced

ADDENDUM

At the time of going to press S V *Boyden* has briefly remarked in the discussion following the presentation of a paper on the role of complement in phagocytosis (Boyden S V North R J & Faulkner S M Complement and the activity of phagocytes p 216 in Complement a Ciba Foundation Symposium ed by Wolstenholme C E W Churchill Ltd London 1965) that phagocytosis occurs in the serum of patients with agammaglobulinaemia

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even washed leucocytes may retain plasma opsonins on their surface, this *observation* seems to cast doubt upon the necessity for interaction with plasma opsonins

Probably the characteristics of the phagocytizing cell itself are what controls the phagocytosis of "inert" particles Thus the rate of ingestion of carbon particles by leucocytes suspended in serum is considerably higher than that for quartz particles (1) The results of quantitative kinetic studies have been interpreted as showing that the latter depends upon the condition of the cells (1)

The present investigation shows conclusively that no antibody like plasma factor is required for neutrophiles to phagocytize iron powder This, however, cannot be concluded with absolute certainty from the results of experiments with the leucocytes suspended in the patients' own plasma since patients with "acquired" hypogammaglobulinaemia may retain a small antibody-producing capacity (8, 9, 10) The statement is instead based on the fact that washed leucocytes also phagocytize iron powder

The clearance of bacteria differs from that of "inert" particles in possessing an element of specificity Specific antibodies are no doubt the most effective of all opsonins (reviews 11, 12, 13) One would expect the opsonization of bacteria to be seriously impaired in hypogammaglobulinaemic conditions, in spite of some findings that only small amounts of antibody appear to be required for opsonization *in vivo* (review 13) This is further evidenced by the diminished though not abolished phagocytosis seen in germ-free animals (14) The results of the present trials with *Staphylococcus aureus* contradict this hypothesis, as the degree of bacterial engulfment was similar by neutrophiles from patients with primary "acquired hypogammaglobulinaemia and from controls It may be taken for granted that normal adults have been immunized against this germ Notably, however phagocytosis took place only in an environment of plasma No phagocytosis occurred in physiological saline, probably due to the absence of certain ions (review 15) or to the fact that a well-washed leucocyte is hardly a physiological cell Hence it has been concluded that the level of other uncharacteristic opsonin-like serum factors—unspecific immunity mechanisms—determine the susceptibility of an individual normal animal and its species to infection by a particular antigen (15, 16) In this respect much importance has been ascribed to the complement or its components (reviews 11, 17), although it has been questioned whether these substances participate in phagocytosis (18) Interestingly several workers have noted no obvious abnormalities in the levels of complement or its components in hypogammaglobulinaemia (19, 20, 21) Indeed it is more plausible that in "acquired" hypogammaglobulinaemia cellular factors rather than humoral are involved in immunity reactions Other observations support this contention Thus it has been feasible to sensitize patients with this disease against strong allergens and to

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STUDIES IN ORAL LEUKOPLAKIAS

9 *Alkaline and Acid Phosphatase in Normal and Leukoplakic Epithelium before and after Vitamin A Application*

By

G Renstrup, F A v Bülow and F P Clausen

Received 4 v 65

The literature contains few publications dealing with histochemical analysis of the content and distribution of alkaline and acid phosphatase in normal and leukoplakic oral mucosa

Previous Investigations

Alkaline phosphatase could not be demonstrated in epithelium from clinically normal or inflamed gingival mucosa by *Zander* (1941) or by *Cabrini & Carranza* (1958) both using the lead nitrate method according to Gomori However, *Lizanti* (1960) demonstrated, by use of an azo coupling technique, great activity in the basal layers of the epithelium from inflamed gingival tissue *Svoboda, Lojda & Skach* (1959) applied both the lead nitrate method according to Gomori and an azo-coupling technique to normal and pathologic oral mucosa from different areas They found inconsistently alkaline phosphatase activity

concentration of alkaline phosphatase is located in the connective tissue in relation to the capillaries and to some collagenous fibrils

Acid phosphatase activity has been described to occur mainly in the epithelial layers of the oral mucosa *Cabrini & Carranza* (1958) used Gomori's lead nitrate method on biopsies from normal and inflamed gingiva and found the greatest acid phosphatase activity in the layers immediately subjacent to the keratin layer, with a gradual diminution toward the basal cell layer They found the keratin layer acid phosphatase free but in parakeratinized epithelium activity was also seen

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cannot be reversed by removing obvious irritants, and 3) cannot be classified clinically or microscopically as another diagnosable disease, (*Silverman et al* 1963 III, VII)

Hyperorthokeratosis is a pathological condition in which the superficial layers of the epithelium are keratinized containing no nuclei and appearing homogenous and strongly acidophilic In areas where orthokeratinization normally occurs it has to exceed the thickness of the control section to be called hyperorthokeratosis

Hyperparakeratosis is a pathological condition in which the outer cell layers are flattened, contain pyknotic nuclei and exhibit strong acidophilia In areas where parakeratinization normally occurs, it has to exceed the thickness of the control section to be called hyperparakeratosis In this study a distinction is made between hyperparakeratosis *with* a stratum granulosum and hyperparakeratosis *without* a stratum granulosum

MATERIAL AND METHODS

Fifteen patients (10 men and 5 women) with oral leukoplakia were studied Their

cheek and
in 4 cases
the end of

III VII) as
... vitamin A acetate troches Ten troches per day for 2 to 3 weeks were used The patients were instructed to place a troche until it was dissolved against the lesion to be studied

Local infiltration anaesthesia was used and care was taken not to inject directly
sies were taken and immediately
The tissue was sectioned at 10 µ

... phosphatase was demonstrated by the method of *Burstone* (1962) For demonstrating *acid* phosphatase two methods were used the lead nitrate reaction by *Gomori* (1958) and the reaction by *Barka* (1963) using hexazonium pararosanilin The two methods gave almost identical results In the few cases of ...

Haematoxylin and eosin stained sections of all biopsies served to determine the type and degree *of keratinization*

RESULTS

Th

su

ke ... Histologically the leukoplakic lesions could be divided into three groups based on the type of keratin ...

... ulosum

Alkaline phosphatase.—The reaction for *alkaline* phosphatase was negative in the epithelium in all sections both from normal mucosa and

in the surface layer. In non-keratinized epithelium there was a great reduction in acid phosphatase activity in all layers. They concluded that there seemed to be a relation between acid phosphatase and keratinization. *Svoboda et al.* (1959) found almost the same distribution of acid phosphatase in the epithelium. They applied the Gomori lead nitrate method as well as an azo-coupling technique to normal and pathological tissue from different areas of the oral mucosa. No acid phosphatase activity could be demonstrated in the keratin layer, however, epithelium with *parakeratosis showed positive reaction in* the surface layer as well. In all remaining layers of epithelium they found acid phosphatase activity. On the contrary, *Lisanti* (1960) found the most intense reaction for acid phosphatase to be localized in the keratinized surface layer of the epithelium from inflamed gingival tissue treated with an azo-coupling technique. Further, he observed a decreasing concentration of acid phosphatase from the superficial to the basal layer. *Ten Cate* (1963), also using both the azo-coupling technique and the lead nitrate method by Gomori on non-keratinized oral mucosa from young monkeys and on gingival tissue from humans, demonstrated acid phosphatase activity to be localized intracellularly in small granules. Ten Cate has interpreted the granules as lysosomes. He stated that, in non-keratinized, parakeratinized and keratinized oral mucosa the granular sites of acid phosphatase activity in stratum germinativum and stratum spinosum were distributed mainly at the cell perifery and perinuclearly. In the superficial cells of non-keratinized epithelium the granular sites were large and distributed irregularly throughout the cells. In the stratum corneum of parakeratinized and keratinized epithelium only a few granular sites of activity were found, but enzyme activity was here demonstrated as a diffuse colour reaction. Ten Cate concluded that the formation of keratin is an active process rather than a degenerative change.

Although nothing definite is known about the significance of alkaline and acid phosphatase in the epithelium in the process of keratinization, it is suggested that there is a relation between the acid phosphatase activity and the keratinization. Clinical and histological studies have shown that the keratinization pattern in oral leukoplakias can be altered by topical application of vitamin A, (*Silverman, Renstrup & Pindborg* 1963). Thus it seems of interest whether vitamin A had any effect on the correlation between the phosphatase activity and the type of keratinization. The present investigation was *undertaken to demonstrate* possible differences in content and distribution of alkaline and acid phosphatase in oral leukoplakias and in adjacent normal oral mucosa before and at *the end of topical vitamin A application*.

Definitions

Leukoplakia in this paper is defined as any white patch *or plaque* on the oral mucous membrane that 1) cannot be removed by scraping, 2)

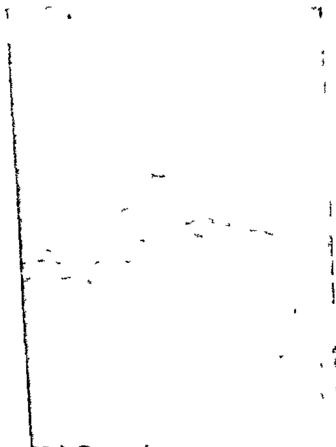


Fig 2

Epithelium without a stratum granulosum (clinical normal oral mucosa parakeratinized) The picture shows a low activity of acid phosphatase throughout the epithelium with a culmination in the basal and middle part of stratum spinosum Orig mag 360 ×

to the stratum basale No activity could be seen in the stratum corneum (Table 1)

The epithelium characterized by *hyperparakeratosis without* a stratum granulosum (group 3) from leukoplakias, and *parakeratosis* from normal oral mucosa, showed equal activity and distribution of acid phosphatase A slight reaction could be demonstrated throughout the epithelium with the highest activity being in the basal and middle layer of the stratum spinosum Fig 2, and no or very low activity in the surface layers (Table 1)

Non keratinized epithelium from normal oral mucosa also showed activity in all the layers of the epithelium though somewhat weaker, and also with a tendency to increase basally (Table 1)

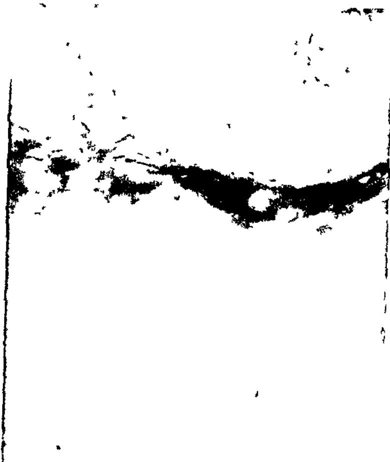


Fig 1

Epithelium with a stratum granulosum (oral leukoplakia hyperorthokeratotic) The activity of acid phosphatase is high in the stratum granulosum decreasing through the stratum spinosum and very low in stratum basale No activity is seen in stratum corneum Orig mag 360 ×

from leukoplakic lesions A positive reaction was found only in the walls of the capillaries where is was quite strong All controls were negative

Acid phosphatase—The *hyperorthokeratotic* epithelium (group 1) of the leukoplakias demonstrated low activity in the stratum basale and the stratum spinosum, whereas the stratum granulosum showed a very high activity, Fig 1 The stratum corneum was without activity (Table 1).

In the leukoplakias demonstrating *hyperparakeratotic* epithelium *with* a stratum granulosum (group 2) the distribution of acid phosphatase was almost the same as in the first type The activity was high in the stratum granulosum, and decreased through the stratum spinosum

DISCUSSION

The results of this investigation concerning *alkaline* phosphatase activity are in agreement with *Zander* (1941) and *Cabrini & Carranza* (1958) who found that the epithelium was free of this enzyme and that there was a strong reaction in the underlying connective tissue in relation to capillaries *Lisanti* (1960) found great activity of alkaline phosphatase in the basal layer of the epithelium probably due to the heavy subepithelial inflammation in his material According to *Montagna & Ellis* (1962) any local disorder will immediately cause an appearance of variable amounts of alkaline phosphatase in the epithelium

Concerning the *acid* phosphatase activity in oral epithelium it is difficult to compare the results of this study with the results obtained by other investigators because of differences in materials and methods and because of different purposes of the studies Nevertheless the results here reported are in agreement with *Cabrini & Carranza*'s (1958) findings of highest enzyme activity immediately subjacent to the keratin layer no activity in the stratum corneum and a gradual decrease basally The last finding was also observed by *Svoboda et al* (1959) The relationship between keratin formation and acid phosphatase activity as found by *Ten Cate* (1963) was confirmed by our findings *Montagna & Ellis* (1962) stated for normal skin that basal cells in epithelium show practically no reaction for acid phosphatase whereas the cells of the stratum spinosum show a gradual increase toward the surface The entire granular layer and the cells immediately above have a very intense reaction even the stratum corneum may be strongly positive The acid phosphatase activity and distribution pattern in keratinizing epithelium from oral leukoplakias as observed in this study can be compared with that of normal skin However we did not see acid phosphatase activity in the stratum corneum in any case of keratinizing oral mucosa The fact that vitamin A has no primary influence on the distribution and activity of acid phosphatase may indicate that the enzyme is not localized in lysosomes As vitamin A is known to cause a liberation of lysosomal acid phosphatase (*Dingle* 1963) it may be concluded that the acid phosphatase of the normal or keratinized oral epithelium is not bound to lysosomes but is found free in the cytoplasm

SUMMARY

Biopsies from 15 leukoplakic lesions and adjacent normal oral mucosa were taken before and after topical application of vitamin A The tissue ... hatase

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The acid phosphatase activity in the epithelium studied, thus demonstrated two types of distribution patterns 1) a pattern characteristic of epithelium *without* a stratum granulosum (non keratinizing epithelium and para- and hyperparakeratinizing epithelium *without* a stratum granulosum) (Fig 2), and 2) a pattern characteristic of epithelium *with a* stratum granulosum (hyperorthokeratinizing and hyperparakeratinizing epithelium *with* a stratum granulosum) (Fig 1) In both patterns the acid phosphatase showed an equally weak activity in the basal and middle part of stratum spinosum, where the activity culminated for the epithelium *without* a stratum granulosum, and then decreased and finally disappeared in the surface layers In contrast, the epithelium *with* a stratum granulosum showed an increasing activity throughout the stratum spinosum with a maximum in the stratum granulosum, and thereafter a complete disappearance of activity in the stratum corneum (Table 1)

The acid phosphatase activity and distribution were solely dependent upon the type of keratinization When a stratum granulosum was present in the epithelium the activity was very strong here, otherwise the epithelium showed a weak acid phosphatase activity The vitamin A application *per se* had no influence on this pattern However, if the vitamin A changed the type of keratinization from a pattern *with* to a pattern *without* a stratum granulosum, the acid phosphatase activity changed according to the type of keratinization Controls of both the lead nitrate reaction by Gomori and the hexazonium pararosanilin reaction by Barka were all negative

TABLE 1

A Graduated Evaluation of the Distribution of Acid Phosphatase in the Epithelium

	Clinically normal epithelium		Leukoplakic epithelium		
	Non kera tin ized	*Para* kera tin ized	Hyperpara *keratosis without* Stratum granulosum	Hyperpara *keratosis with* Stratum granulosum	Hyperortho keratosis
Keratin layer		—	—	—	—
Stratum *granulosum*				++++	++++
Upper stratum spinosum	+	+	+	+++	+++
Medium stratum spinosum	++	++	++	++	++
Lower stratum spinosum	++	++	++	++	++
Basal layer	+	+	+	+	+

DISCUSSION

The results of this investigation concerning *alkaline* phosphatase activity are in agreement with *Zander* (1941) and *Cabrini & Carranza* (1958) who found that the epithelium was free of this enzyme and that there was a strong reaction in the underlying connective tissue in relation to capillaries *Lisanti* (1960) found great activity of alkaline phosphatase in the basal layer of the epithelium probably due to the heavy subepithelial inflammation in his material According to *Montagna & Ellis* (1962) any local disorder will immediately cause an appearance of variable amounts of alkaline phosphatase in the epithelium

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SUMMARY

Biopsies from 15 leukoplakic lesions and adjacent normal oral mucosa were taken before and after topical application of vitamin A The tissue was stained for alkaline and acid phosphatase

Alkaline phosphatase could not be demonstrated in the epithelium Based on the distribution pattern of *acid phosphatase* in the epithelium the biopsy material could be divided into two groups 1) char

acterized by the presence of a stratum granulosum with high activity and 2) characterized by the absence of a stratum granulosum, with a low activity in the basal and middle part of stratum spinosum Vitamin A administration in itself had no direct influence on the acid phosphatase distribution

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ULTRASTRUCTURE OF THE RAT THYMUS

By

P M Lundin and U Schelin

Received 8 v 65

Since the thymus was decribed in detail at the end of the 19th century its position and function in relation to other lymphoid organs has been a matter of controversy Most investigators agree that the small lymphocyte of the thymus sometimes called thymocyte, is morphologically indistinguishable from the small lymphocyte in the spleen and lymph nodes but it has been known for a very long time that the thymus often reacts differently from other lymphoid organs It was early observed that during manition the involution of the thymus is much more rapid than that of spleen and lymph nodes That this rapid involution is an unspecific reaction depending on the stimulation of the pituitary adrenal axis has been made clear by the extensive work of Selye and collaborators (see for example *Selye* 1950)

The development of the thymus from the outgrowth of epithelial tubes from the third pharyngeal pouches is known in detail as is the derivation from these tubes of the epithelial cells in the thymus But the relation between the epithelial cells and the lymphocytes has been discussed at length Until recently the common concept was that the lymphocytes were derived from the mesenchyme and that some of the reticular cells in the thymus were of mesenchymal origin (cf *Baillif* 1949) These problems have been reinvestigated during the last few years partly with newer techniques Thus *Ball & Auerbach* (1960) showed that embryonal mouse thymus cultured at a glass clot interface remained epithelial but when transplanted to the anterior eye chamber of isologous recipients it became lymphoid With the hanging drop technique they could prove that the presence of mesenchyme was essential for the development of lymphoid tissue When the epithelial anlage was separated from the mesenchyme by a millipore filter barrier the epithelium developed into lymphoid tissue (cf *Auerbach* 1961)

The problem of the identification of cell types in the thymus is suitable for an electron microscopic approach Recently a few papers have been published where the results of electron microscopic studies on the thymus especially that of the mouse have been presented *Hoshino* (1962) was first to describe the ultrastr

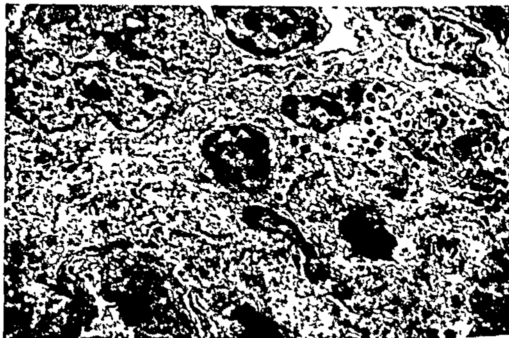


Fig 1
Portion of the thymic capsule with two capillaries (CAP) and a mast cell (MC) × 3,500

infiltrated with lymphoid cells and perforated by reticular fibers and blood vessels A basement membrane separated the epithelial cells from the connective tissue He presumed that the epithelium was derived from the ecto-entoderm He could find no signs that epithelial cells produced lymphocytes Large macrophages contained numerous inclusions but possessed none of the distinctive features of epithelial cells *Weiss* (1963) studied the reticular cells in the thymus, and found the epithelial nature of them evident by the existence of tonofibrils and desmosomes After intravenous injection thorium dioxide may be found in the vessel wall and, to a limited degree, in the surrounding tissue

In spite of the last years' extensive work there are many problems left and there is no general agreement about the origin, fate and function of the thymocyte The four questions drawn up as early as 1949 by *Baillif* still seem to remain unanswered 1 The identity of the thymocyte Is the thymocyte derived from the epithelium and does the morphological likeness establish an identity with the lymphocyte? 2 The fate of the thymocyte, especially during involution 3 The components of the thymic structure Is the thymus framework double in nature, being formed both from the mesoderm and the epithelium? 4 The thymic macrophage Is it related to the epithelial reticular cells?

As a base for our studies of the histology and ultrastructure of the thymus during cortisone-induced involution, the ultrastructure of the normal adult rat thymus has been investigated and the results are reported here

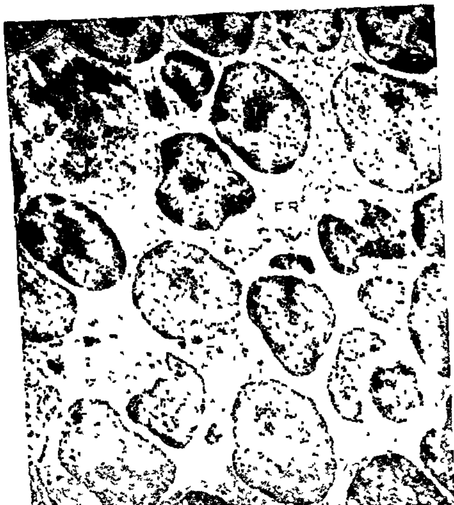


Fig 2

Survey picture from the cortex The lymphocyte (L) is the dominating cell type The lymphocyte in the upper left shows a mitotic figure FR part of an epithelial reticular cell with tonofibrils in the cytoplasm × 5 700

MATERIALS AND METHODS

The observations to be reported here were based on the examination of the thymus from male Sprague Dawley rats weighing 200–250 g Under ether anaesthesia some of the animals were injected intravenously with 0 1–2 0 ml of an [illegible]

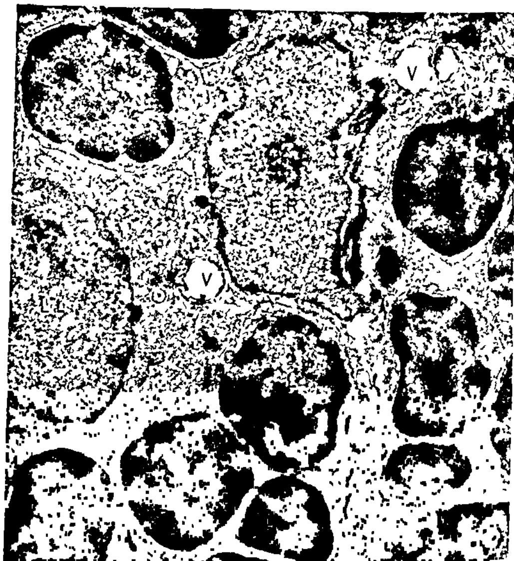


Fig 3

Survey picture from the medulla In the centre an epithelial reticular cell (ER) with vacuoles (V) and tonofibrils (T) in the cytoplasm To the left a large lymphocyte (LL) with finely dispersed nuclear chromatin L, lymphocytes × 6 600

stained with uranyl acetate (*Watson* 1958) or lead (*Millonig* 1961) Acid phosphatase was demonstrated with the technique described by *Miller* (1962)

For light microscopy the tissue was fixed in formalin embedded in paraffin, and stained by Dominici's method (*Romeis* 1948) and for iron by a modified Prussian blue reaction (*Hutchison* 1953) The Epon embedded thick sections were stained with toluidine blue (*Bjorkman* 1962)

RESULTS

By electron microscopy the thymus is seen to be sourrounded by a thin capsule (Fig 1) and to contain a delicate fibrous stroma penetrating the parenchyma There is a sparse network of small vessels concentrated more in the medulla than in the cortex The thymic paren-



Fig 4

A small vessel lined by a thick endothelium (END) showing cytoplasmic processes Outside the endothelium there is a perivascular space containing collagen fibrils (C) Fr ep thel al reticular cells with tonofibrils and cytoplasmic inclusions L lymphocytes × 10 500

chyma consists of lymphoid cells and two types of cells with a nuclear structure characteristic for reticular cells We call these cells epithelial reticular cells and mesenchymal reticular cells (macrophages) according to *Downey* (1948) In the cortex reticular cells are sparse (Fig 2) but in the medulla both types of reticular cells are more numerous and often much larger (Fig 3)

The lymphoid cells of the thymus are separated from the small blood vessels by epithelial reticular cells and their basement membrane Thus the barrier between the blood and lymphoid tissue is composed of the endothelium with its basement membrane a perivascular space occasionally containing collagen fibrils and pericytes and epithelial reticular cells with their basement membrane (Figs 4 and 5)

In the normal thymus lymphocytes of various sizes are the most numerous cells (Figs 2 and 3) Their appearance is essentially that of lymphocytes elsewhere (*Clark* 1962) Sometimes lymphocytes with large nuclei and a chromatin structure resembling that of epithelial

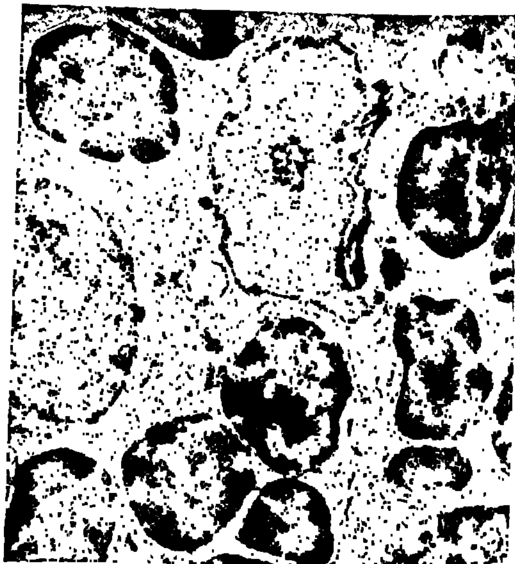


Fig 3

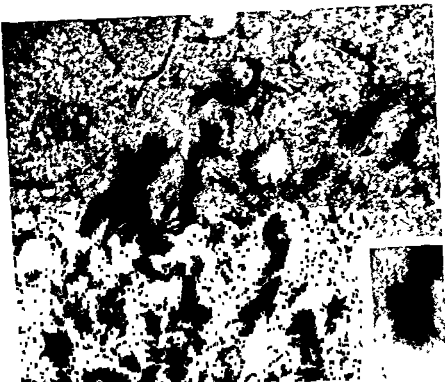
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Figs 6 and 7 (inset)

Fig 6 Part of an epithelial reticular cell showing the tonofibrils of the cytoplasm × 23,000

Fig 7 Desmosome between adjacent epithelial reticular cells × 86,000

sections stained for acid phosphatase (Fig 13) Within the cytoplasm in some of the macrophages there are small lymphocytes in varying stages of degeneration (Fig 14)

In sections stained for iron, the thymus from the animals injected with iron-dextran shows in the light microscope granules within macrophages in the outer zone of the medulla In the electron microscope, mesenchymal reticular cells are found in the perivascular space, but some of them appear in the thymic parenchyma (Fig 15) The iron-dextran is present in the cytoplasm of the mesenchymal reticular cells in the form of electron-dense aggregates (Fig 16) of the type seen in macrophages after iron dextran injection (*Daems* 1962)

DISCUSSION

The structure of the thymus as seen in the light microscope has been well known since *Hammar's* work (1921, 1936) Besides the vessels and the perivascular stroma the thymus consists of lymphocytes and

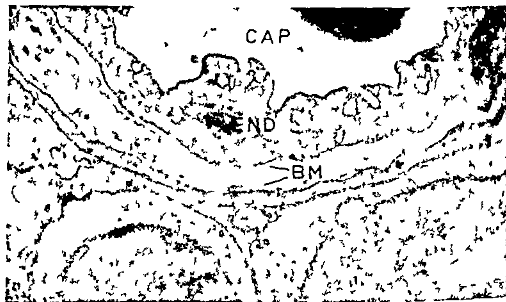


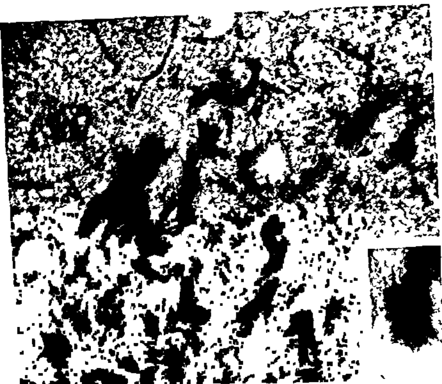
Fig 5

Detail from a capillary showing the basement membranes (BM) in the perivascular space CAP capillary lumen END endothelium × 21 000

reticular cells can be observed but their cytoplasm is scant and without tonofibrils and desmosomes (Fig 3) Mitotic figures are fairly frequent (Fig 2) Small lymphocytes are often seen with electron dense pyenotic nuclei probably undergoing degeneration Occasional plasma cells (Fig 11), eosinophils and mast cells (Fig 1) are found especially near the surface of the cortex or around blood vessels

Epithelial reticular cells are of the same appearance in the cortex as in the medulla They can be identified by their tonofibrils and desmosomes connecting adjacent epithelial reticular cells (Figs 6 and 7) Desmosomes between epithelial reticular cells and lymphocytes are not observed The nuclei are large with a finely dispersed chromatin and prominent nucleoli (Fig 3) The epithelial reticular cells characteristically show cytoplasmic vacuoles (Figs 3 8 and 9) The walls of the vacuoles are sometimes seen to have microvillous projections (Fig 8) and the lumina contain structureless material of moderate density Sometimes the cytoplasm of the epithelial reticular cells also contains inclusions in the form of lipid droplets or rounded dense granules (Fig 9) These inclusions are negative in sections stained for acid phosphatase

Mesenchymal reticular cells (macrophages) are encountered both in the cortex and the medulla but are more numerous in the latter They possess nuclei similar to the epithelial reticular cells (Figs 10 11 and 14), but differ from them in lacking desmosomes and tonofibrils The mesenchymal reticular cells are filled with numerous inclusions of varying sizes and densities (Figs 10 11 and 12) Many of the inclusions are vacuolated (Fig 12) and many of them show positive reactions in



Figs 6 and 7 (inset)

Fig 6 Part of an epithelial reticular cell showing the tonofibrils of the cytoplasm × 23 000

Fig 7 Desmosome between adjacent epithelial reticular cells × 86 000

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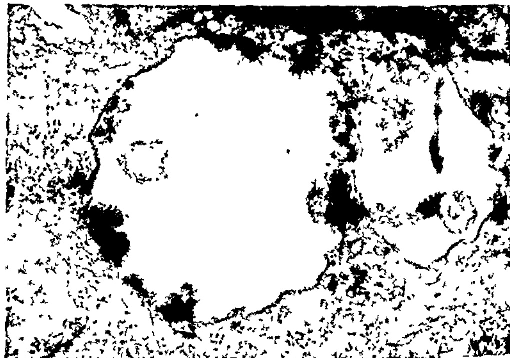


Fig 8
Cytoplasmic vacuoles with "microvillous" projections in an epithelial reticular cell × 50,000

reticular cells The lymphoid cells are morphologically identical with lymphocytes in lymph nodes and other lymphatic tissues, but their absolute identity with other lymphocytes has been denied by some authors and they are sometimes called thymocytes The reticular cells are larger and elongated with pale nuclei and one or two nucleoli The epithelial nature of most of the reticular cells, especially in the medulla, is hardly questioned nowadays, but the problems of mesenchymal reticular cells and the relation between reticular cells and lymphocytopoiesis are still much discussed *Downey* (1948) differentiated between epithelial reticular cells and mesenchymal reticular cells by their differences in the nuclear structures and the occurrence of many small vacuoles in the cytoplasm of the epithelial cell In addition, phagocytosed material could be found in the mesenchymal cells From differential counts of resting and dividing cells, *Leblond & Sainte-Marie* (1960) proposed a model for lymphocyte formation, in which the small lymphocyte is the ultimate cell in a series of eight successive generations from the reticular cells and large lymphocytes through smaller and smaller lymphocytes In the medulla, however, it appeared that the evolution of the reticular cells into lymphocytes was the exception, rather than the rule as in the cortex They admit that the reticular cells may not be a homogenous population, there may be two kinds and only one type may be capable of phagocytosis The medullary inclusion cells described by *Ito* (1959) in the hamster and the chromolipoid cells in

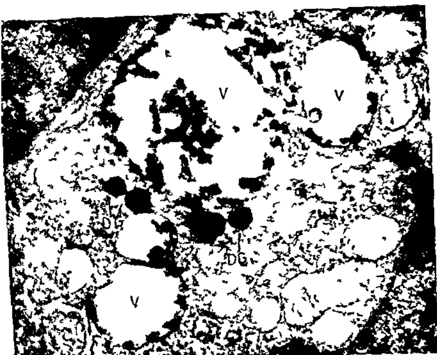


Fig 9
Part of an epithelial reticular cell with vacuoles (V) and dense granules (DG) × 23 000

the cortex of ageing mice seem to be special types of mesenchymal reticular cells (*Loewenthal & Smith* 1952)

Thus difficulties in differentiating in the light microscope between the two types of reticular cells are apparent In the electron microscope however the two types of cells are easily distinguished

Our results concerning the ultrastructure of the different cell types their distribution in cortex and medulla and their relation to the vessels are fundamentally in agreement with the results obtained by *Clark* (1963) and *Weiss* (1963) from their studies of the mouse thymus In accordance with *Clark* (1963) we could find no signs of direct transformation of epithelial cells to lymphoid cells either in the cortex or in the medulla But some of the most immature lymphoid cells showed a chromatin structure resembling that of the epithelial cells *Weiss* (1963) describes only one type of reticular cells and concludes that they are phagocytic and may contain fat He finds the epithelial nature of many reticular cells evident by tonofilaments desmosomes and cysts *Clark* (1963) on the other hand speaks of macrophages containing numerous inclusions but possessing none of the distinctive features of epithelial cells Thus they have neither desmosomes nor tonofibrils Our results are in agreement with *Clark*s (1963) observations on the

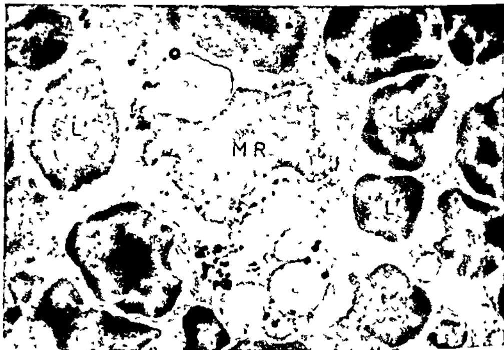
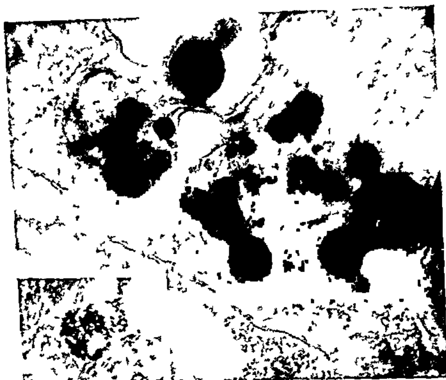


Fig 10
Mesenchymal reticular cell (MR) with cytoplasmic inclusions of varying densities L, lymphocytes × 6,000

Fig 11
A mesenchymal reticular cell (MR) with cytoplasmic inclusions and a plasma cell (PC) from the outer zone of the medulla × 6,500



Figs 12 and 13 (inset)

Fig 12 Cytoplasmic inclus ons in a mesenchymal reticular cell. Some of them contain vacuoles × 53 000

Fig 13 Cytoplasmis inclus on w th pos t ve react on for acid phosphatase × 40 000

possibility of differentiation in the normal thymus between the typical epithelial cell and the macrophage and we have found the same types of inclusion bodies in the cytoplasm of the macrophages Many of these inclusion bodies showed the structure descril ed at characteristic for lysosomes (see for example *Novil off* 1963) and in addition they showed positive reactions for acid phosphatase Correlated biochemical and morphological studies have earlier been reported suggesting the lysosomal nature of "dense bodies from thymus (cf *deDuve* 1963)

Nakamura & Metcalf (1961) have pointed out that the incidence of pycnotic lymphocytes usually exceeds the numl er of mitoses in the normal thymus Likewise we have found that it is very easy to find lymphocytes with pycnotic nuclei in the electron microscope and that many macrophages have phagocytosed degenerating lymphocytes This must suggest a continuous lymphocytolysis in the normal thymus As we are ignorant of the duration of the degenerating process of a lymphocyte it is impossible to estimate the percentage of lymphoid cells undergoing lysis within the thymic tissue

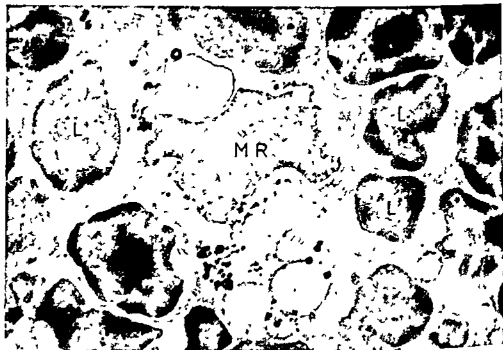


Fig 10.

Mesenchymal reticular cell (MR) with cytoplasmic inclusions of varying densities L, lymphocytes × 6,000.

Fig 11

A mesenchymal reticular cell (MR) with cytoplasmic inclusions and a plasma cell (PC) from the outer zone of the medulla × 6 500



Fig 15

Part of the outer zone of the medulla from an animal injected with iron-dextran with macrophages containing electron-dense aggregates in the cytoplasm (MR1, MR2, MR3) Two of them (MR1, MR2) lie in the perivascular space, one of them (MR3) among lymphocytes (L) in the thymic parenchyma CAP; capillary C, perivascular space with collagen fibrils. × 3,800

from penetrating into the thymic parenchyma *Weiss* (1963 b) found that the enclosure about the cortical vessels was incomplete, and Thorotrast injected intravenously may be seen extravascularly. Our results demonstrate that this barrier at least in some part of the marrow can be passed by a substance with a molecular weight of about 200,000.



Fig 14
Mesenchymal reticular cell with degenerating lymphocytes in the cytoplasm × 5 500

None of the earlier authors has discussed the relation between the epithelium and the stroma cells We have found that some of the macrophages are situated in the perivascular tissue between the vessel wall and the epithelium, but many lay in the parenchyma among the lymphoid cells, especially in the medulla But compared to other organs belonging to the reticulo-endothelial (RES) system the normal thymus certainly has few macrophages

After intravenous administration of macromolecular substances such as iron-dextrin and iron-dextran, only negligible amounts are taken up in the thymus compared to the spleen, liver and lymph nodes We have been able to confirm this with the aid of Fe^{59} labelled iron-dextrin (unpublished observations) In the latter organs most of the macromolecular substances are phagocytosed by littoral cells in the walls of lymph node sinuses and the sinusoids of the liver The thymus lacks such littoral cells, and this seems to be the reason why such small amounts of these substances are taken up in the thymic tissue

Clark (1963) has postulated that the epithelial cells with their basement membrane, which form a continuous layer around the vessels, represent a haemothymic barrier that prevents antigens and vital dyes

stained for acid phosphatase Many of the macrophages contain degenerated lymphocytes suggesting a continuous lymphocytolysis

Injected iron dextran is found in macrophages near the vessels in the outer zone of the medulla Some of the macrophages are found in the perivascular space but some of them appear in the thymic parenchyma suggesting that the thymic barrier at least in some part of the marrow can be passed by a substance with a molecular weight of about 200 000

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Fig 16
Iron containing aggregate in a macrophage from an animal injected with iron dextran × 41 000

SUMMARY

The ultrastructure of the normal adult rat thymus has been studied. The thymic parenchyma consists of three main types of cells: lymphoid cells, epithelial reticular cells and mesenchymal reticular cells (macrophages). The parenchyma is separated from the small blood vessels by a barrier which consists of the endothelial cells with their basement membrane, a perivascular space occasionally containing collagen fibrils and pericytes, and the epithelial reticular cells with their basement membrane.

Lymphocytes are the most numerous cells with the same appearance as lymphocytes elsewhere. There are no signs that lymphoid cells are formed from epithelial cells but sometimes lymphocytes are observed whose large nuclei have a chromatin structure resembling that of epithelial cells.

The epithelial reticular cells can be identified by their tonofibrils, desmosomes, cytoplasmic ciliated vacuoles and large nuclei with a finely dispersed chromatin.

Mesenchymal reticular cells (macrophages) possess nuclei similar to the epithelial reticular cells but differ from them in lacking desmosomes and tonofibrils. The mesenchymal reticular cells contain inclusions of varying sizes and densities. Some of these inclusions show a fine structure characteristic for lysosomes and are positive in sections

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ACTION OF HEPARIN AND PLASMINOGEN INHIBITOR (EACA) ON METASTATIC TUMOUR SPREAD IN AN ISOLOGOUS SYSTEM

By

BERNT BOERYD

Received 23 xi 65

It has been shown experimentally that various exogenous factors are capable of influencing the ability of circulating tumour cells to develop into metastases Thus the metastasis rate to liver and lungs has been modified in both homologous (2, 6, 7, 8, 9, 13, 20, 36) and isologous systems (24, 32, 33, 35) by alteration in coagulability and fibrinolytic activity of the blood

Wood's studies *in vivo* (34) on the pathogenesis of tumour metastases from V 2 carcinoma in a rabbit ear chamber disclosed that the initial stages of adhesion of the tumour cells to the endothelial lining of vessels were associated with the formation of thrombi around the tumour cells [illegible]

[illegible] carcino- [illegible] treatment with human fibrinolysin Further *Fisher* & *Fisher* (10) noted fewer and smaller metastases to the liver in heparin treated rats after intraportal inoculation of Walker [illegible]

Conversely, diminis [illegible]

[illegible] another type of tumour [illegible] ascitic V 2 carcinoma cells intravenously inoculated into rabbits *Wood* (36) found [illegible]

This investigation was supported by research grants from the *Swedish Cancer Society*

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second group comprised control mice given 0 75 ml of a 7 5 per cent aqueous NaCl solution by stomach tube about 90 minutes prior to inoculation with tumour cells to check the effect of fluid given to the EACA group below, the colloidosmotic pressure exerted by 7 5 per cent NaCl solution being of the same order as that exerted by a 30 per cent EACA solution The third group received 0 05 ml of a 1 per cent Heparin solution (Vitrum, Stockholm) intraperitoneally 15 to 20 minutes before inoculation with tumour cell
capillary method, was prolonged to
detected for at least two hours The
EACA solution (Kabi, Stockholm) b
with tumour cells

The effect of EACA on the fibrinolytic system was tested by determining the lysis time for whole blood coagulum using the principle of the streptokinase resistance test (17, 23) Fibrinolysis was induced with urokinase because streptokinase proved ineffective on mouse blood Into tubes containing 0 05 ml of urokinase (Leo pharmaceutical products, Denmark 600 Ploug Units per ml physiological saline) was pipetted 0 1 ml of citrated blood (4 parts blood + 1 part sodium citrate) immediately followed by 0 05 ml of thrombin Roche (Topostasin 100 NIH Units per ml sterile water), whereupon the tubes were placed in a water bath at 37° The tubes were lifted out and tilted every 5 minutes to check the progress of coagulolysis Coagulolysis was complete in normal blood within 10 minutes in 100 per cent of the trials From each of 14 mice 0 15 ml of blood was drawn from the jugular vein at different lengths of time after administration of 0 75 ml of a 30 per cent EACA solution, and 0 1 ml was used for testing the fibrinolysis inhibition as described above The fibrinolysis was markedly inhibited in all the mice between 1½ and 8 hours after EACA administration (clot lysis time >6 hours) No EACA activity could be detected after 10 hours

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Series A comprising 49 mice, was divided into three groups—a control group, a group pretreated with heparin and a group pretreated with EACA like the corre
00 cells
EACA
00 cells
second
day after inoculation were discarded

Series B comprising 40 mice was divided into three groups—a control group, 5 mice of which were pretreated with 0 1 ml of physiological saline intravenously, the oth
1 per c … ith 0 05 ml
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… inhibited fibrinolysis for over 1 hour (clot lysis time >

The

monstrated that after continuous heparin treatment rabbits given an intravenous inoculation of a comminuted suspension of V 2 carcinoma cells had fewer and grossly smaller metastases to lungs but simultaneously an increased number of metastases to several other organs

On the other hand *Retil et al* (24) have reported an increased number of spontaneous metastases to the lungs in an isologous system following prolonged heparin treatment of C57 Bl 6JN mice inoculated intramuscularly with T 241 sarcoma

The manner in which the anticoagulants exert their presumably metastasis inhibiting action remains an open question, by (i) cytotoxic effect, or (ii) by preventing or delaying the adhesion of the tumour cells and or the formation of a fibrin coagulum around them which could be essential for the initiation of a growth focus of viable tumour cells in the vascular system, or (iii) by redistribution of these cells to localities less suitable for tumour growth than the lungs

Thornes reported that various fibrinolytic agents may inhibit *in vitro* the growth of HeLa cancer cells (29) *Lisnell & Mellgren* (16) obtained the same result with streptokinase, dicoumarol and protamine On the other hand *Lisnell & Mellgren* demonstrated that heparin is not directly cytotoxic and also that epsilon-amino-n-caproic acid (EACA), an inhibitor of plasminogen activation *in vitro* (28) and in man (21) and also an antidote to induced fibrinolysis in animals (22), is cytotoxic only in very high concentration and not growth promoting *in vitro*

Therefore it was decided to test the effect of heparin and EACA on the incidence, size and distribution of tumour metastases in an isologous system The significance of the type of cell suspension was tested using one mechanically produced cell suspension and another produced by enzymatic activity

MATERIALS AND METHODS

The experiments were made on a line of CBA mice inbred from progenitors supplied in 1960 by *G Klein* of the Department of Tumour Biology Stockholm The tumour was a rhabdomyosarcoma (MCG 1) induced by 20 methylcholantrene in female mice of the same CBA strain (*Mellgren et al* (19) to be published) After inoculation of the sarcoma in the groin of CBA mice there were 100 per cent "takes" and spontaneous metastases to the lungs and occasionally to other organs

Two cell suspensions were used a mechanically and an enzymatically produced type The mechanically produced suspension was prepared from about 2 g of finely minced tumour tissue stirred with 10 ml of Hank s BSS in a Magni Mixer at 37° C for 15 minutes Following filtration through a nylon filter with 28 µ pores a suspension was obtained which microscopically contained a few per cent of aggregates with 2 to 5 cells the remainder beeing single cells Cell viability was estimated with the aid of vital staining and incorporation of tritiated thymidine and adenine (*Boeryd et al* (4)) the proportion of viable cells being from 8 to 10 per cent

The enzymatically produced cell suspension was prepared according to *Mad len & Burk* (18), with some modifications *viz* (a) the stock solution was Morgan 150 (b) the last hour s stirring was carried out manually at 5 minute intervals and (c) the cells were concentrated by centrifugation at 100 g for 10 minutes Microscopically this gave a suspension of exclusively single cells containing about 90 per cent viable cells as determined by the methods just mentioned *The cells were* counted in a Celloscope (AB L Ljungberg Stockholm) calibrated with a Burker cell count

The mechanical cell suspension was inoculated in a series of 36 mice divided into four groups The first group comprised controls receiving *no pretreatment* The

gross metastases other than to the liver there were no differences between the groups Mice in Series B disclosed at autopsy grossly visible metastases to the liver and in some mice to the kidneys, adrenals, mesenterium, lymph nodes and subcutis Subcutaneous metastases were present in 8 of 11 heparin-treated mice and in only 3 mice from each of the two other groups With respect to the other gross metastases there were no differences between the groups

TABLE 1

Pulmonary Metastases Induced by Mechanically Produced Cell Suspension

All mice					Mice with metastases				
	No of mice	N	$V \cdot 10^{-6}$	$V \cdot 10^{-3}$		No of mice	N	$V \cdot 10^{-6}$	$V \cdot 10^{-3}$
Controls	16	145	16	2 3	Controls	7	304	16	4 9
Heparin	6	57	8	0 5	Heparin	2	159	8	1 2
EACA	9	65	40	2 6	EACA	6	94	40	3 8

TABLE 2

Metastases Induced by Enzymatically Produced Cell Suspension Series A

All mice to lungs					Mice with metastases to lungs				
	No of mice	N	$V \cdot 10^{-6}$	$V \cdot 10^{-3}$		No of mice	N	$V \cdot 10^{-6}$	$V \cdot 10^{-3}$
Controls	15	127	25	3	Controls	13	146	25	4
Heparin	9	26	84	2	Heparin	4	71	85	6
EACA	13	57	511	29	EACA	12	62	509	31
to liver					**to liver**				
	No of mice	N	$V \cdot 10^{-6}$	$V \cdot 10^{-3}$		No of mice	N	$V \cdot 10^{-6}$	$V \cdot 10^{-3}$
Controls	15	25	9560	240	Controls	14	26	9520	249
Heparin	9	51	3380	174	Heparin	8	57	3400	195
EACA	13	13	8450	106	EACA	10	16	8510	133

As appears from Tables 2 and 3, the tendencies in Series A and B are similar in the following respects When all the animals with and without metastases are considered the heparinized ones showed a reduction in the number of metastases to the lungs and also an increase in the

Provided all metastases *are spherical* and of equal volume within each separate animal then the below formulae for calculating the total volume of the animals *metastases and the number and average volume of individual metastases are valid*

$$V = a \qquad (1)$$

$$N = \sqrt{\frac{\pi}{6}} \; \frac{n^{3/2}}{\sqrt{a}} \qquad (2)$$

$$\bar{v} = \sqrt{\frac{6}{\pi}} \; \left(\frac{a}{n}\right)^{3} \qquad (3)$$

where

V = total metastasis volume in cm^3 per cm^3 organ tissue
N = *number of metastases per cm^3 organ tissue*
n = observed number of metastases per cm^2 organ tissue
a = total area in cm^2 of all metastases observed per cm^2 organ tissue and
$\bar{v}$ = average volume of a single metastasis in cm^3

Formula (1) still gives a good approximation even if the individual animal's metastases have unequal volumes and different shapes provided they *remain randomly* distributed and disposed within the organ and have convex shapes These conditions *are satisfied at least to a very* close approximation by metastases in the present investigation

In calculating N from Formula (2) the fact that the metastases have unequal *volumes will of course* be a source of error Samples have shown however that the *metastases to the lungs as well as those to the liver had closely conforming fre*quency distribution curves in the various experimental groups Accordingly in the present investigation it was justified to use N as an estimate of the true number *of metastases* $\bar{N}$ *In the aforementioned paper (5) it is demonstrated that under certain reasonable assumptions* $N < \bar{N} < 1.4\,N$ *and also that in this inequality* N may be considered as a lower limit for $\bar{N}$ Analogously since $\bar{v}$ is a function of V and N the reasoning applying to N is also valid for $\bar{v}$ in the present investigation

The results in the tables are given in the form of group means for V N and $\bar{v}$ the *means being calculated from group means for a and n Differences between* groups were tested by Wilcoxon's two sample rank test and with Chi square analysis according to Fisher A difference with $p < 0.05$ was accepted as significant

RESULTS

In the mice inoculated with *mechanical cell suspension* no metastases were grossly visible at autopsy in any organ No differences appeared between untreated and NaCl treated mice inoculated with the aforementioned cell suspension and they will henceforth be considered as a single group (Table 1) Histologically no liver metastases were encountered Admittedly the differences were not statistically significant but in the heparin treated group the number of pulmonary takes was slightly smaller and the average number and total area of metastases to the lungs were smaller than in the controls and the lung metastases in the FACA-treated group seemed fewer on the average but larger No attempts were made to obtain more reliable figures by further experimentation since mechanical cell suspension seemed unsuited for continued study

Enzymatic suspension At autopsy of mice in series A metastases were grossly visible in the liver and occasionally in the *kidneys adre*nals, mesenterium, ovaries, myocardium and subcutis With respect to

ference, evidently owing to skew distribution) at the same time as their average volume had increased (178 ∓ 26, $P < 0.02$ by Chi square)

Heparinization increased the number and reduced the mean volume of the metastases to the liver compared with the controls, whether all mice or only those with metastases to the liver were considered (all differences $P < 0.05$ according to Wilcoxon) On the other hand, the incidence of takes in the liver remained unchanged after heparin administration, as apparently did the total metastasis volume per cm^3 liver tissue in mice with takes

TABLE 4

Series A and B

	All mice to lungs				*Mice with metastases* to lungs			
	No of mice	N	v 10^6	V 10^3	No of mice	N	v 10^6	V 10^3
Controls	29	114	21	2	20	154	26	4
Heparin	20	41	178	8	10	88	178	16
EACA	26	93	227	21	24	103	226	23

	to liver				to liver			
	No of mice	N	v 10^6	V 10^3	No of mice	N	v 10^6	V 10^3
Controls	29	27	6170	169	28	29	6140	179
Heparin	20	54	2430	130	18	60	2430	146
EACA	26	16	5250	82	22	19	5220	100

EACA treatment tended to increase the incidence of takes in the lungs but the differences compared with controls (24/26 and 20/29) are not significant The same applies to Series B

When all mice even when only those with metastases to the lungs, are considered, the numbers of metastases seemed to be reduced, but these tendencies are not significant On the other hand, the total metastasis volume in the lungs was found to be increased in all animals in the total material and also if only those with takes in the lungs are considered (21 ∓ 2 and 23 ∓ 4, $P < 0.05$ in both cases according to Wilcoxon) also the mean metastasis volume was found to have increased (227 ∓ 21, $P < 0.02$ and 226 ∓ 26, $P < 0.05$, respectively, both determined by Chi-square)

EACA treatment did not affect the incidence of takes in the liver The number and total volume of metastases tended to be smaller

number of metastases to the liver Moreover the mean volume of the metastases seemed to increase in the lungs and to diminish in the liver The EACA-treated mice showed tendencies to increased total and average metastasis volumes in the lungs and to a decrease in the number of metastases to the liver When only animals with metastases are considered identical tendencies appeared Statistical analysis of the data within each series yields no significant differences, but as both series showed similar tendencies it was deemed justified to combine them and subject the consolidated data to statistical analysis See Table 4

TABLE 3

Metastases Induced by Enzymatically Produced Cell Suspension Series B

	All mice to lungs					*Mice with metastases* to lungs			
	No of mice	N	$v \times 10^{-6}$	$V \times 10^{-3}$		No of mice	N	$v \times 10^{-6}$	$V \times 10^{-3}$
Controls	14	101	13	1	Controls	7	220	13	3
Heparin	11	66	213	14	Heparin	6	101	214	22
FACA	13	210	44	9	FACA	12	240	44	11

	to liver					to liver			
	No of mice	N	$v \times 10^{-6}$	$V \times 10^{-3}$		No of mice	N	$v \times 10^{-6}$	$V \times 10^{-3}$
Controls	14	34	2510	86	Controls	14	34	2510	86
Heparin	11	59	1460	86	Heparin	10	66	1460	97
EACA	13	23	2080	48	EACA	12	27	2080	57

Heparinization produced an apparent reduction in the incidence of takes in the lungs, but compared with the controls (10/20 and 20 29 respectively) the difference was not statistically significant Nor was there such a difference in Series B

When all mice in the experimental groups are compared, whether they had metastases to the lungs or not, the number of metastases per cm³ of tissue admittedly seemed reduced and the total and average metastasis volumes increased These differences are just as little significant, however, as in the separate series, evidently owing to considerable dispersion Conversely, if only mice with takes in the lungs are considered, the number of metastases per cm³ tissue proved significantly lower in the heparinized mice than in the controls (88 ∓ 154, P < 0 05 according to Wilcoxon, but the Chisquare test yields no significant dif-

The diverging results obtained with the two cell suspensions with respect to metastatic distribution seem to illustrate the importance of using a highly viable single cell suspension for metastatic experiments.

The results of these experiments should cast some doubt on the advisability of the clinical use of heparin as an agent against the formation of new metastases as suggested by some authors (2).

SUMMARY

Heparinized and Epsilon amino n caproic acid treated mice have been inoculated intravenously with either a mechanically or an enzymatically produced cell suspension of a 20 methylcholanthrene induced rhabdomyosarcoma (MCG 1) in an isologous system. With the enzymatically produced cell suspension heparin inhibited the number of pulmonary metastases but increased the number of liver metastases while EACA increased the total volume of pulmonary metastases.

In this tumour host system heparin and the antifibrinolysin seem essentially to affect transpulmonary passage of tumour cells. The results suggest that in this system thrombosis is not essential for metastatic growth.

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DISCUSSION

The results observed after inoculation of mechanically produced cell suspension into heparin-treated mice bear out experiments reported by previous workers, especially the experiments reported by *Wood et al* (32, 33, 35) who used similar suspensions of C-150 tumour cells in Swiss mice

After intravenous inoculation of the highly viable separate cell suspension into heparin-treated isologous mice, heparin seems to make such tumour cells in this tumour-host system more liable to pass the lung and to grow in other organs, among them the liver and the subcutis Of course vital cells might well reach other organs as well but these would escape detection with this mode of study and relatively short observation time The results obtained in EACA-treated mice suggest that the main effect of inhibition of plasminogen activity is to reinforce the sieve action of the lungs, retaining more cells there

The different effects of EACA-treatment on the number of metastases to the lungs in Series A and B presumably has some relation to the much larger dose given in Series A As demonstrated by Lisnell and Mellgren EACA exerts a cytotoxic action in a concentrations exceeding 2500 mg per 100 ml, a level possibly attained at least temporarily in Series A

The behaviour of liver metastases in heparin as well as EACA-treated mice suggests that at least during the first hours after tumour cell dissemination in this system, thrombosis is not essential for metastatic growth (including penetration and establishment) Of course, this does not rule out that coagulum formation may promote the growth of metastases, either through aggregation of more cells at one site or by facilitating adhesion, penetration, and establishment

These results seem to fit well with Selecki's and Ambrus' findings Thus *Selecki* (27) reported a constant release of tumour cells from the lungs for up to 24 hours after intravenous inoculation of Cr 51 labelled Ehrlich ascites tumour cells in normal mice In heparin-treated Webster Swiss mice inoculated with Ehrlich ascites tumour cells *Ambrus et al* (3) found increased transpulmonary passage of tumour cells, or at least delayed fixation in other organs

The passage of tumour cells in any system through the lungs under influence of heparin is perhaps not due just to loss of stickness by fibrin Another haemodynamic effect of heparin possibly could be deduced from the literature Thus heparin is supposed to decrease erythrocyte aggregation and, in the light of work by Fåhraeus and his school on the haemodynamic effect of erythrocyte aggregation, it may be assumed that heparin causes the large malignant cells to travel in the axial current, perhaps making them more liable to pass the lungs and end up in other organs such as the spleen where they might be destroyed (11, 12, 30) or where small, scattered metastases might have escaped detection

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COMPLEMENT-FIXING LIVER ANTIBODIES

1 *Occurrence in a Material of Normal Blood Donors*

By

G Norup

Received 6 iv 65

Several investigations have been carried out in patients suffering from acute and chronic hepatitis (cirrhosis hepatis) in order to determine whether a production of antibodies against the patient's own liver occurs

Fiessinger (1908) demonstrated the presence of complement fixing antibodies to liver tissue in sera from 12 out of 15 patients with nutritional hepatitis (Laennec's cirrhosis), whereas sera from a control material consisting of 4 patients with diseases not primarily affecting the liver contained no such antibodies Fiessinger used normal human liver obtained at necropsy as antigen, and a certain post-mortem autolysis may have occurred, although the liver was removed as soon as possible after death

Using normal as well as pathological liver obtained at necropsy as antigen, *Eaton, Murphy & Hanford* (1944) found complement-fixing antibodies in sera from 67 out of 222 (30 2 per cent) patients with infective hepatitis and hepatitis resulting from inoculation with yellow fever vaccine However, in a material consisting of 56 patients with primary atypical pneumonia, complement fixing liver antibodies were demonstrated in 11 (19 6 per cent) of the cases, and sera from 11 of 142 (7 7 per cent) normals similarly showed a positive reaction with the above-mentioned antigens

Later in 1944, *Findlay, Martin & Mitchell* reported that an extract of liver from a fatal case of acute yellow atrophy fixed complement in the presence of sera from patients with infectious hepatitis (10 out of 11) as well as from patients convalescent from this disease (5 out of 5) On the other hand, with extracts of normal human liver obtained at necropsy as antigen all the above sera were negative Neither of the two antigens reacted with sera from a control material consisting of 5 hospitalized patients chosen at random Of these, one had jaundice which developed during treatment with neoarsphenamine injections

These results prompted *Bjørneboe & Krag* (1947) to examine sera from patients with acute and chronic hepatitis for complement fixing

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COMPLEMENT-FIXING LIVER ANTIBODIES

1 *Occurrence in a Material of Normal Blood Donors*

By

G NORUP

Received 6 iv 65

Several investigations have been carried out in patients suffering from acute and chronic hepatitis (cirrhosis hepatis) in order to determine whether a production of antibodies against the patient's own liver occurs

Fiessinger (1908) demonstrated the presence of complement-fixing antibodies to liver tissue in sera from 12 out of 15 patients with nutritional hepatitis (Laennec's cirrhosis), whereas sera from a control material consisting of 4 patients with diseases not primarily affecting the liver contained no such antibodies Fiessinger used normal human liver obtained at necropsy as antigen, and a certain post-mortem autolysis may have occurred, although the liver was removed as soon as possible after death

Using normal as well as pathological liver obtained at necropsy as antigen, *Eaton, Murphy & Hanford* (1944) found complement fixing antibodies in sera from 67 out of 222 (30 2 per cent) patients with infective hepatitis and hepatitis resulting from inoculation with yellow fever vaccine However, in a material consisting of 56 patients with primary atypical pneumonia, complement fixing liver antibodies were demonstrated in 11 (19 6 per cent) of the cases, and sera from 11 of 142 (7 7 per cent) normals similarly showed a positive reaction with the above-mentioned antigens

Later in 1944, *Findlay, Martin & Mitchell* reported that an extract of liver from a fatal case of acute yellow atrophy fixed complement in the presence of sera from patients with infectious hepatitis (10 out of 11) as well as from patients convalescent from this disease (5 out of 5) On the other hand, with extracts of normal human liver obtained at necropsy as antigen all the above sera were negative Neither of the two antigens reacted with sera from a control material consisting of 5 hospitalized patients chosen at random Of these, one had jaundice which developed during treatment with neoarsphenamine injections

These results prompted *Bjorneboe & Krag* (1947) to examine sera from patients with acute and chronic hepatitis for complement fixing

antibodies against an extract of liver from a patient who had died from acute yellow atrophy of the liver. With this antigen, the complement fixation test was positive in 10 of 51 (19.6 per cent) sera from patients with acute hepatitis, and in 8 of 26 (30.8 per cent) sera from patients with chronic hepatitis. The same result was, however, found with extracts of normal human liver, kidney and myocardium as well as with extracts of normal liver and kidney from rabbits and guinea pigs. The complement-fixing antibodies were thus neither organ nor species-specific, an observation which has later been confirmed by *Vorlaender* (1952) and *Gajdusek* (1957). In a control material consisting of sera from 259 individuals, *Bjorneboe & Krag* (1947) found 8 (3.1 per cent) to be positive with the above antigens. The sera of this material were from hospitalized patients with no clinical symptoms of liver disease and with a negative Wassermann reaction[1].

Since, however, it has been described that circulating antibodies against various tissue homogenates may occur in apparently normal individuals after a virus infection (*Eaton et al.* 1944), and in familial predisposition to systemic lupus erythematosus (*Morteo et al.* 1961), in rheumatoid arthritis (*Ziff et al.* 1958), and in Hashimoto's thyroiditis (*Hall et al.* 1960), it would have been of interest if the control materials of the above-mentioned investigations had also been questioned about these conditions.

In all the studies referred to above, the antigens were prepared from liver tissue obtained at necropsy, but since necropsy liver must be assumed to be less suitable for use as the antigen owing to the post-mortem autolysis, the extent of which will be dependent on a number of variable factors (*e.g.* the time elapsing from death occurs until the excision of the tissue, the condition of the patient at the time of death, the temperature of the surroundings, etc.), it was considered of interest to perform a complement fixation test on a material of sera from healthy individuals using as antigen extracts of liver tissue excised during laparotomy, by which procedure the above-mentioned variable factors are eliminated.

MATERIALS AND METHODS

Sera. A total of 278 sera were examined. Of these, 238 were derived from blood donors, 20 from pregnant women in their last month of pregnancy, and 20 from new born babies. Sera were stored at −20° C, and were thawed under tap water before testing.

By questioning the blood donors, the pregnant women, and the mothers of the new born babies, it was ensured that there were no diseases of the liver or biliary tract, no thyroid diseases, rheumatic diseases, renal diseases, and allergic diseases, and, finally, that they had all been in good health during the last three months.

Of the 238 blood donors, 149 were males and 89 females. 131 were aged 18–40, 54 were 41–50, and 53 were 51–65 old. All but one of the pregnant women were under 40 years of age. Of the 20 new born babies, 9 were boys and 11 girls.

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1 Personal communication.

The antigens used were 32 preparations of the following human and animal

rhesus monkeys Finally 5) thyroid tissue from 2 patients suffering from thyroto-
cosis (operation biopsies) was included

Dependent on the amount of antigen available a larger or smaller number of sera from the donor material was tested with each of the 32 antigen preparations Each serum was tested with at least one portion of the antigens 1, 4 and 5 mentioned above

The complement used for the complement fixation test was guinea pig serum, prepared according to the method of *Richardson* (1941)

Haemolytic system (amboceptor + sheep red cells) To the amboceptor prepared by immunization of rabbits with a suspension of sheep red cell stroma, was added glycerine in equal parts The mixture was stored at —20° C Normal sheep cells were prepared from blood which was collected under sterile conditions in bottles con-

nd sub-
+4° C
distilled

The complement fixation test was based on a modification of *Donnelley*'s (1951) semi micro method described by *Gajdusek* (1958) In accordance with this method

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tr ... in amounts of 0 5–1 0 ml in small tubes and stored at —20° C. At this temperature no appreciable loss of complement fixing activity was observed during 5–6 weeks After this time the activity gradually diminished while the anticomplementary effect of the extract increased

Sheep red cells The sheep red cells were washed three times in diluted Veronal saline buffer, and subsequently a 6 per cent cell suspension was prepared and standardized spectrophotometrically at 541 mμ From this a 2 per cent cell sus-
pen

tu

add ... cells to a two per cent

The tray was shaken and incubated for 30 minutes at 37° C whereafter the titration was read

The maximum dilution of the complement giving total (100 per cent) haemolysis of the sheep red cells added, contains one 100 per cent haemolytic unit of complement For the experiment proper two 100 per cent haemolytic units were used

Complement was titrated in the same way in the presence of antigen one drop of the diluent being replaced by one drop of the antigen dilutions which were to be used in the experiment of the day

Antigen titration Before an antigen was used its complement fixing effect in the presence of some positive reference sera was determined by a chess board titration In this titration a series of antigen dilutions was allowed to react with a series of serum dilutions together with a constant quantity of complement The dilutions of serum and antigen were made in Wassermann tubes while the experiment was performed in a haemagglutination tray To each cup was added one drop of serum dilution one drop of complement dilution containing two 100 per cent haemolytic units per drop and one drop of antigen dilution The mixture was incubated for one hour at 37° C and subsequently one drop of the haemolytic system was added The tray was then shaken and incubated for 30 minutes at 37° C with a further shaking after 15 minutes The readings could then be performed The highest concentration of the antigen giving complete fixation of the added complement with the lowest concentration of serum was used in the following experiments In the case of antigens prepared from human liver obtained at operation and liver from rhesus monkeys this antigen concentration was rather constant from one preparation to the other corresponding to 25 35 mg of raw liver tissue per one ml of antigen suspension

Performance of test Serum was inactivated at 56° C for 30 minutes at a 1 4 dilution in Veronal saline buffer before testing With a dropper pipette serial two fold dilutions 1 8 1 16 1 512 were prepared from the primary dilution 1 4 by adding 5 drops of the serum dilution to 5 drops of Veronal saline buffer in a cup of a haemagglutination tray After mixing the cup was sucked dry, to this cup was added one drop and to the next cup 5 drops of the serum dilution Each serum was at the same time tested with extracts of liver and thyroid as well as examined for anticomplementary effect To each serum dilution was added one drop of a complement dilution containing two 100 per cent haemolytic units per drop and one drop of the antigen dilution After thorough shaking the haemagglutination tray was incubated for one hour at 37° C Then one drop of the sensitized sheep cells was added to each cup and after shaking the haemagglutination tray was incubated for 30 minutes at 37° C with a further shaking after 15 minutes In order to facilitate the evaluation of the haemolysis percentages the haemagglutination tray was stored at +4° C for 4–6 hours to precipitate the unlysed cells The haemolysis percentages were read with the naked eye and were indicated by the figures from 0–4 0 representing 100 per cent haemolysis 1 2 and 3 75 50 and 25 per cent haemolysis respectively and 4 no haemolysis With practice a comparison with standards was found unnecessary The titre of a serum was read as the reciprocal of the highest serum dilution giving the value 2, representing about 50 per cent haemolysis Only titres of 8 or more were regarded as positive since titres of 4 were found quite frequently and at repeated determinations were inconstantly present

For the purpose of comparing the results obtained on different days at least 2 positive sera the titres of which had been determinated on several previous occasions were included in all experiments ment of the

later communication)

RESULTS

As shown in Table 1 seven out of 238 sera (2 9 per cent) from normal blood bank donors were positive with antigens prepared from normal human liver obtained at biopsy as well as with antigens from liver of rhesus monkeys, and six out of the same 238 sera (2 5 per cent) gave

a positive test with extracts of human thyrotoxic glands obtained at operation (Two of the above seven reactive sera and four non-reactive sera)

TABLE 1

Results of Complement Fixation Test with Liver and Thyroid Antigens on 238 Normal Blood Bank Donors *

Tissues	No of preparations	No of sera tested	No of sera positive	Per centage positive	137 sera tested with all antigen types: No of sera positive	137 sera tested with all antigen types: Per centage
Normal human livers (biopsy)	11	238	7	2 9	5	3 7
Normal livers from rhesus monkeys	10	238	7	2 9	5	3 7
Normal human livers (necropsy)	3	138	7	–	7	5 1
Normal human foetal livers	6	150	0	–	0	0 0
Thyrotoxic human thyroid glands (biopsy)	2	238	6	2 5	6	4 4

* All sera from 20 pregnant women and 20 new born babies were negative

Of 138 sera tested with normal human liver obtained at necropsy seven were positive Of these, four sera had previously given a positive reaction with human biopsy liver and liver from rhesus monkeys None of the 150 sera tested with extracts of human foetal liver were positive, although eight sera positive with adult human biopsy liver and/or liver obtained at necropsy were included A total of 137 sera were tested with all the antigen types (Table 1) As it appears from this reduced material a higher frequency of positive tests, expressed in percentages, was found with extracts of normal human necropsy liver (5 1 per cent) than with fresh liver obtained surgically from patients and from rhesus monkeys (3 7 per cent) Although the figures are too small for definite conclusions, the result nevertheless suggests an antigenic difference between necropsy liver and fresh liver

A total of 14 out of 238 sera were positive with one or more of the antigens shown in Table 2

It will appear from this table that two sera (Nos 1 and 10) gave a positive test with antigens from both liver and thyroid tissue, 8 sera (Nos 2 9) were positive with antigens from liver tissue only, and 4 sera (Nos 11–14) with antigens from thyroid tissue only Of the 10 sera (Nos 1–10 in *Table 2*) which gave a positive complement fixation test with antigens from liver tissue, 4 (Nos 1–4) reacted with fresh normal liver obtained surgically from patients and from rhesus monkeys as

well as with normal necropsy liver, 3 sera (Nos 5–7) reacted only with the two first-mentioned antigens, and, finally, 3 sera (Nos 8–10) were positive with human necropsy liver only

TABLE 2

Titre of Complement Fixing Antibody of 14 Positive Sera Tested with Antigens from Liver and Thyroid Tissues *

Serial No	Age (yr)	Sex	Normal liver tissue: Human biopsy	Normal liver tissue: Human necropsy	Normal liver tissue: Rhesus monkey	Thyrotoxic Thyroid glands: Human biopsy
1	48	♂	16	16	8	8
3	32	♂	8	16	8	0
2 }	63	♀	8	8	8	0
4 }	44	♂				
5	20	♂	16	0	16	0
6	52	♀	8	0	8	0
7	38	♀	8	n t §	8	0
10	48	♂	0	8	0	8
8	56	♀	0	16	0	0
9	57	♀	0	8	0	0
13	57	♀	0	0	0	16
11 }	44	♂	0	0	0	8
12 }	51	♀				
14 }	42	♀				

* All 14 sera except nos 6 and 7 were tested with human foetal liver All were negative

§ Not tested

TABLE 3

Distribution of Positive Tests on Sex and Age of the Blood Bank Donors

Age group (yrs)	Men: Total tested	Men: No of positive	Women: Total tested	Women: No of positive
18–40	99	2	32	1
41–50	32	4	22	1
51–65	18	0	35	6
	149	6	89	8

The difference between the frequencies of positive tests in donors over and under 40 years was not statistically significant

The distribution of positive reactions on sex and age of the blood bank donors is shown in Table 3

It can be seen that a positive complement fixation test was found in six men and eight women 11 out of 14 individuals with a positive test were more than 40 years old Of these, 5 (4 men and 1 woman) were aged 41–50, and 6 (all women) aged 51–60

DISCUSSION

With the modification of *Donnelley's* (1951) semi-micro method used in the present study, complement-fixing antibodies to fresh normal human liver tissue were demonstrated in seven out of 238 (2 9 per cent) healthy blood bank donors

These results are in good agreement with those obtained by *Bjorneboe & Krag* (1947) and *Gajdusek* (1958), but contrast the findings of *Eaton et al* (1944) and *Hackett et al* (1960) The differences may to some extent reflect fluctuations in the level of sensitivity but may also be due to qualitative differences in the antigens used, especially if these are prepared from tissues obtained at necropsy Of interest in this regard is an observation made by *Vorlaender* (1957), in attempting to produce antibodies against homologous liver tissue in rabbits, he found that the organ specificity was lost if the liver tissue had undergone autolysis more than 48 hours prior to the injection into the animal

In the present study the antigens prepared from necropsy liver were found less suitable than those prepared from biopsy material Thus four of seven livers obtained at necropsy could not be used, mainly as a result of a marked anticomplementary effect, and considerable variations in the complement fixing activity were found from one preparation to the other, an observation which has also been made by *Beall* (1963)

It seems likely that the relatively poor agreement between the results obtained with biopsy liver and autopsy liver may be ascribed to the post-mortem autolysis of the last mentioned tissue This assumption is further supported by the complete accordance in antigenicity between human biopsy liver and liver from rhesus monkeys which were excised in almost the same way

When the results of the various investigations are compared another observation that should be considered is the composition of the examined groups as far as sex and age are concerned Thus *Hackett et al* (1960) have shown that complement fixing antibodies to various tissues are more frequent in women than in men in the decades below 60 years, while above this age the frequency of positive tests was equal in the two sexes

In this investigation a positive complement fixation test to liver and thyroid antigens was demonstrated more frequently in females than in males aged 50 65 years, but the difference between the frequencies was not statistically significant

SUMMARY

With a modification of Donnelley's semi micro method, complement fixing antibodies against fresh human liver and liver from rhesus monkeys were demonstrated in 2 9 per cent of sera from a material of healthy blood bank donors

well as with normal necropsy liver, 3 sera (Nos 5–7) reacted only with the two first-mentioned antigens, and, finally, 3 sera (Nos 8–10) were positive with human necropsy liver only

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STUDIES ON PURIFIED C 1 ESTERASE AND HEREDITARY ANGIONEUROTIC OEDEMA SERA BY COMBINED ELECTROPHORESIS AND IMMUNE HAEMOLYSIS

By

R SIBOO[1] and A B LAURELL

Received 30 iv 65

The first component of complement (C 1) was reported to be converted to an active enzyme by antigen antibody complexes (*Lepow et al* 1954) Additional experimental evidence supported this finding when *Levine* (1955) and *Becker* (1956a) found that diisopropyl fluorophosphate an inhibitor of a number of esterases, inhibited C 1 activity in the presence but not in the absence of sensitized sheep erythrocytes (EA) *Becker* (1956b) observed that EAC 1 4 2 cells would hydrolyze p toluene sulphonyl L arginine methyl ester (TAMe) and proposed that C 1 existed in serum as a proesterase which was converted to an esterase during the course of immune haemolysis *Lepow and associates* (1956a and 1956b) also reported that a partially purified preparation of C 1 and a factor eluted from an antigen antibody C complex hydrolyzed N acetyl L-tyrosine ethyl ester (ATEe)

Recent chromatographic studies by *Lepow et al* (1963) showed that C 1 could be fractionated into three subcomponents (C 1q C 1r and C 1s) A more highly purified preparation of C 1 esterase was obtained by chromatography of activated englobulin (*Haines & Lepow* 1964 a) The C 1 esterase activity was shown to be related to C 1s and it inactivated C 4 and C 2 and hydrolyzed ATEe and other synthetic esters The most highly purified preparation of C 1 esterase, however was found to contain at least two proteins by ultracentrifugation and immunoelectrophoretic techniques (*Haines & Lepow* (1964 b c)

The activity of C 1 esterase was shown by *Pensky et al* (1961) to be inhibited by an inhibitor of C 1 esterase in normal sera

Donaldson and associates (1963 1964) reported that sera from affected members of families with hereditary angioneurotic oedema inactivated C 4 C 2 and hydrolyzed ATEe They concluded that C 1 ester

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[1] Medical Research Fellow Medical Research Council of Canada

This investigation was supported by grants from the *Swedish Medical Research Council* and from the *Alfred Österlunds Foundation*

In the material were only included sera from persons who after questioning in detail must be assumed to have been in good health during the preceding three months and who had no history of diseases of the liver, the biliary tract, the thyroid, nor of rheumatic renal or allergic diseases

The results obtained with antigens from biopsy liver differed to some extent from those obtained with necropsy liver

A non significant accumulation of reactivity was found for males aged 40–50 years and for females aged 50 65 years

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STUDIES ON PURIFIED C'1 ESTERASE AND HEREDITARY ANGIONEUROTIC OEDEMA SERA BY COMBINED ELECTROPHORESIS AND IMMUNE HAEMOLYSIS

By

R SIBOO[1] and A -B LAURELL

Received 30 iv 65

The first component of complement (C'1) was reported to be converted to an active enzyme by antigen antibody complexes (*Lepow et al* 1954) Additional experimental evidence supported this finding when *Levine* (1955) and *Becker* (1956 a) found that diisopropyl fluorophosphate, an inhibitor of a number of esterases inhibited C'1 activity in the presence but not in the absence of sensitized sheep erythrocytes (EA) *Becker* (1956 b) observed that EAC'1,4,2 cells would hydrolyze p toluene sulphonyl L-arginine methyl ester (TAMe) and proposed that C'1 existed in serum as a proesterase which was converted to an esterase during the course of immune haemolysis *Lepow and associates* (1956 a and 1956 b) also reported that a partially purified preparation of C'1 and a factor, eluted from an antigen antibody-C' complex, hydrolyzed N-acetyl-L-tyrosine ethyl ester (ATEe)

Recent chromatographic studies by *Lepow et al* (1963) showed that C'1 could be fractionated into three subcomponents (C'1q, C'1r and C'1s) A more highly purified preparation of C'1 esterase was obtained by chromatography of activated englobulin (*Haines & Lepow* 1964 a) The C'1 esterase activity was shown to be related to C'1s, and it inactivated C'4 and C'2 and hydrolyzed ATEe and other synthetic esters The most highly purified preparation of C'1 esterase, however, was found to contain at least two proteins by ultracentrifugation and immunoelectrophoretic techniques (*Haines & Lepow* (1964 b, c)

The activity of C'1 esterase was shown by *Pensky et al* (1961) to be inhibited by an inhibitor of C'1-esterase in normal sera

Donaldson and associates (1963, 1964) reported that sera from affected members of families with hereditary angioneurotic oedema inactivated C'4 C'2 and hydrolyzed ATEe They concluded that C'1 ester-

[1] Medical Research Fellow, Medical Research Council of Canada

This investigation was supported by grants from the Swedish Medical Research Council and from the Alfred Österlunds Foundation

ase existed in the sera of these patients They also reported that these sera lacked C'1 esterase inhibitor

The above studies demonstrated that C'1 esterase could inactivate C components—C'4 and C'2 It has been shown that antibody synthesis by cells could be demonstrated by immune haemolysis in agar gels (*Jerne et al* 1963) Serological haemolysis was carried out also in agar gels (*Milgrom & Millers* 1963) It appeared, therefore, that an electrophoretic technique could be developed to identify C'1 esterase activity by its ability to inactivate C' components This investigation describes a technique, which combines immune haemolysis and electrophoresis in a mixture of EA and agarose as the supporting medium, to demonstrate C'1 esterase activity

MATERIALS AND METHODS

Preparation of C'1 esterase Normal human sera were fractionated by the method of *Haines & Lepow* (1964a) Purification was not continued after the second cycle Preparations of second cycle C'1 esterase were concentrated against carbowax 20 000 or by lyophilization and stored at —60° C

Preparation of C'1 esterase inhibitor Partially purified C'1 esterase inhibitor was fractionated from normal human sera on a Dowex 2 × 10 200 (Dow Chemical Co) column according to *Pensky et al* (1961) The preparations were kept at —60° C

Sera from healthy individuals and from members of family (W) with hereditary angioneurotic oedema Blood from staff members were collected and sera prepared for control purposes Blood samples were collected from affected members of a family (W) during attacks when C'1 esterase activity in serum has been shown to be pronounced (*Donalson & Rosen* 1964) and from unaffected members of the same family Sera were prepared and were stored at —60° C

Tests for C'1 esterase activity The hydrolysis of ATFe by partially purified C'1 esterase and by sera from affected members of a family (W) was determined in a [illegible] of
pH stat [illegible] ml
the C'1 [illegible] hyl
of 5 × [illegible]
cellosolve) The hydrolysis was determined at pH 7 4 and [illegible]

The inactivation of C'4 was carried out according to the principles outlined by *Haines & Lepow* (1964b) C'4 was prepared by the method of *Müller Eberhard & Biro* (1963) The C'4 preparations were used after column chromatography without additional purification by Pevikon electrophoresis

Test for C'1 esterase inhibitor The hydrolysis of ATFe by partially purified C'1 esterase was followed for 7 min as described above and then 0 1 ml of C'1 esterase inhibitor normal sera or sera from the family (W) with hereditary angioneurotic oedema was added to the reaction mixture The hydrolysis of ATFe was followed for another 7 min The inhibitor activity was determined from the slopes of the curves The units of C'1 esterase and C'1 esterase inhibitor are expressed in terms as defined by *Lepow et al* (1963) and *Pensky et al* (1961)

Electrophoretic technique Veronal buffered saline (VB saline) without gelatin and isotonic sucrose veronal buffer (VB sucrose) were prepared according to *Rapp & Borsos* (1963) Veronal saline sucrose (VSS) buffer was prepared by mixing 1 4 [illegible] of VB saline with 3 6 volumes of VB sucrose to give an ionic strength of [illegible] dissolving 1 5 g of agarose [illegible] 100 ml of VSS buffer Sheep [illegible] spended in VSS buffer to a final concentration of 4 per cent

The sheep erythrocytes were sensitized with an equal volume of eight units of haemolysin which was diluted in VSS buffer Melted agarose solution (1 5 per cent) and sensitized erythrocytes (EA) were brought to 44 45° C Equal volumes of agarose and EA were mixed (final concentration of agarose 0 75 per cent and EA 1 0 per cent) and poured on slides coated with a thin agarose film A layer of the supporting

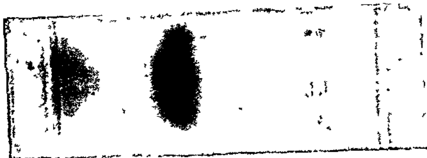


Fig 1

Electrophoresis of partially purified C'1 esterase The zone of unlysed cells is indicative of the mobility of the protein associated with C'1 esterase activity

medium was produced by applying 5 and 10 ml to 2 6 × 12 2 cm and 5.3 × 12 2 cm slides, respectively The electrode vessels contained barbital buffer pH 8 2 $\mu = 0\ 05$ Electrophoresis was carried out at 4° C at a constant current of 0 33 mA per cm The length of time for the electrophoretic separation was determined by the migration of albumin in simultaneously performed control runnings of human serum

[illegible]

plugged with agar The slides were treated with complement as described above It is important to keep the antigen slit and antiserum trough relatively close (4 mm apart) to each other so that the precipitation lines develop rapidly In this way, the precipitation lines as well as the zone of unlysed cells will be evident after incubation at 4° C overnight

The C'1 esterase activity was characterized by a zone of unlysed EA since C'1 esterase destroyed some of the added C components in this area The absence of C'1 esterase in other areas of the slide would result in lysis of the EA by the added rabbit C The electrophoretic mobility of the protein associated with C'1 esterase activity was determined by direct comparison of the zone of unlysed EA with the precipitation lines formed between normal human serum and anti total human serum

RESULTS

Several partially purified C'1 esterase preparations were found to produce the effect as described in the following section One such preparation which contained 636 units ml was used throughout this investigation to establish conditions for the electrophoretic technique C'1 esterase activity was demonstrated by a zone of unlysed cells in the supporting medium (Fig 1) Inhibition of C'1 esterase by inhibitor of C'1 esterase or heating resulted in the absence of the zone

Various dilutions of partially purified C'1 esterase were subjected to electrophoresis The zones of unlysed EA established the activity of the

ase existed in the sera of these patients They also reported that these sera lacked C'1 esterase inhibitor

The above studies demonstrated that C'1 esterase could inactivate C components—C'4 and C 2 It has been shown that antibody synthesis by cells could be demonstrated by immune haemolysis in agar gels (*Jerne et al* 1963) Serological haemolysis was carried out also in agar gels (*Milgrom & Millers* 1963) It appeared, therefore, that an electrophoretic technique could be developed to identify C 1 esterase activity by its ability to inactivate C' components This investigation describes a technique, which combines immune haemolysis and electrophoresis in a mixture of EA and agarose as the supporting medium, to demonstrate C'1 esterase activity

MATERIALS AND METHODS

Preparation of C'1 esterase Normal human sera were fractionated by the method of *Haines & Lepow* (1964a) Purification was not continued after the second cycle Preparations of second cycle C 1 esterase were concentrated against carbowax 20 000 or by lyophilization and stored at —60° C

Preparation of C 1 esterase inhibitor Pa ... tor was fractionated from normal human sera on ... column according to *Pensky et al* (1961)

Sera from healthy individuals and from members of family (W) w... angioneurotic oedema Blood from staff members were collected and sera prepared for control purposes Blood samples were collected from affected members of a family (W) during attacks when C 1 esterase activity in serum has been shown to be pronounced (*Donaldson & Rosen* 1964) and from unaffected members of the same family Sera were prepared and were stored at —60° C

Tests for C'1 esterase activity The hydrolysis of ATEe by partially purified C'1 esterase and by sera from affected members of a family (W) was determined in a pH stat (Radiometer Copenhagen Denmark) The method consisted of incubation of the C 1 esterase preparations or the patients sera—0 5 ml were incubated with 1 9 ml of 5 × 10 3 M phosphate buffer pH 7 4 and 0 1 ml of ATFe (1 0 M ATFe in methyl cellosolve) The hydrolysis was determined at pH 7 4 and 37° C for 15 minutes

The inactivation of C 4 was carried out according to the principles outlined by *Haines & Lepow* (1964b) C 4 was prepared by the method of *Muller Eberhard & Bio* (1963) The C'4 preparations were used after column chromatography without additional purification by Pevikon electrophoresis

Test for C 1 esterase inhibitor The hydrolysis of ATFe by partially purified C'1 esterase was followed for 7 min as described above and then 0 1 ml of C 1 esterase inhibitor normal sera or sera from the family (W with hereditary angioneurotic oedema was added to the reaction mixture The hydrolysis of ATEe was followed for another 7 min The inhibitor activity was determined from the slopes of the curves The units of C 1 esterase and C 1 esterase inhibitor are expressed in terms as defined by *Lepow et al* (1963) and *Pensky et al* (1961)

Electrophoretic technique Veronal buffered saline (VB saline) without gelatin and isotonic sucrose veronal buffer (VB sucrose) were prepared according to *Rapp & Borsos* (1963) Veronal saline sucrose (VSS) buffer was prepared by mixing 1 4 ... of VB saline with 3 6 volumes of VB sucrose to give an ionic strength of ... 1 5 g of agarose ... SS buffer Sheep ... VSS buffer to a ... th an equal volume of eight units of ... Melted agarose solution (1 5 per cent) ... to 44–45° C Equal volumes of agarose and EA were mixed (final concentration of agarose 0 75 per cent and EA 1 0 per cent) and poured on slides coated with a thin agarose film A layer of the supporting

Fig 3

Electrophoresis of purified C 1 esterase and sera from affected and unaffected members of family (W) (1) Serum from affected member (A W) (2) C 1 esterase (1 19 U) (3) Serum from unaffected member (K W) The electrophoretic slides were used directly as negative for photography

produce zones of unlysed cells The electrophoresis of one affected and one unaffected member of family (W) is shown in Fig 3 It would appear therefore ,that C 1 esterase activity in the sera of affected members could be identified by this electrophoretic technique It was also evident in electrophoretic studies of sera from affected patients that the zones of unlysed cells were found to be slightly cathodic to the zones of unlysed cells produced by partially purified C 1 esterase (Fig 3) This phenomenon is under investigation

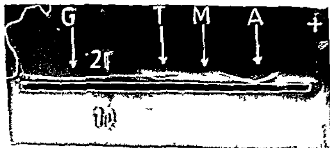


Fig 4



Fig 2
Electrophoresis of various dilutions of a partially purified preparation of C'1 esterase (636 U/ml) 15 µl of each dilution were applied in each slit (1) 1 4 (2 38 U) (2) 1 8 (1 19 U) (3) 1 16 (0 60 U), (4) 1 32 (0 30 U) The zones in the photograph are produced by unlysed cells

C'1 esterase preparations at different dilutions The zones of unlysed cells (Fig 2) clearly show that a concentration of 0 30 units and less can be detected by this technique The method is reproducible and appears to be more sensitive than the esterolytic test with ATEe

The electrophoretic technique was next applied to detect C'1 esterase activity in the sera of patients with hereditary angioneurotic oedema

The properties of sera from three affected and two unaffected members of family (W) are shown in Table 1 These properties are in agreement with those reported by *Donaldson and associates* (1963, 1964)

TABLE 1
Characteristics of Sera from Family (W) with Hereditary Angioneurotic Oedema

Patient	*Clinical symptoms*	*Hydrolysis of ATEe units ml*	*Inhibitor of C'1 esterase units ml*	*C'4 activity in serum*
AW	yes	49 0	0	0
S B W	yes	44 0	0	0
BW	yes	32 0	0	0
KW	no	0	14 0	normal
LW	no	0	15 5	normal

The sera of three affected members produced zones of unlysed cells similar to those produced by partially purified C'1 esterase The sera from two unaffected members as well as normal human sera did not

ported the mobility as an α globulin The technique used in our investigation, however, has the added advantage of demonstrating the enzymatic specificity of the C′1 esterase protein that simple immunoelectrophoresis lacks

All three sera from affected members of family (W) produced zones of unlysed cells in the supporting medium after electrophoresis It was observed, however, that the zones produced by the sera of affected members of family (W) were slightly cathodic to the zones produced with partially purified C 1 esterase *Donaldson & Rosen* (1964) found that the C 1 esterase activity in sera from patients with hereditary angioneurotic oedema was recovered in a sucrose density gradient with protein fractions of high molecular weights They suggested that C′1 esterase activation in the sera of these patients did not require dissociation of the macromolecular complex of C′1 The apparent slower mobility of the C′1 esterase protein in the sera of affected members of family W might be due to the complexing of C′1 esterase with other proteins This problem is under investigation

It seems that the technique described in this investigation could be applied in investigations of anticomplementary factors This would be of advantage in investigations of sera from patients in which one or more C′ components are absent

SUMMARY

A technique which combines electrophoresis and immune haemolysis in a mixture of agarose and EA as the supporting medium was used to detect C′1 esterase enzymatic activity The electrophoretic technique was used to detect C′1 esterase activity in the sera of patients with hereditary angioneurotic oedema The results indicated that the C′1 esterase protein migrated in the zone between the α_1 and α_2 globulins

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Inhibition of C'1 esterase was carried out to determine whether or not the unlysed zone of cells was indicative of C'1 esterase activity. When purified C'1 esterase and serum from patient AW were incubated respectively with C'1 esterase inhibitor prior to electrophoresis no zones of unlysed cells appeared. Similarly, zones of unlysed cells failed to appear when electrophoresis was carried out and inhibitor added to the slide prior to the addition of C'. Heat inactivation of C'1 esterase at 60° C for 30 min or in a boiling water bath for 5 min also resulted in inactivation of the esterase (i.e. no zones of unlysed cells were present). Thus, it would appear that the zones of unlysed cells are indicative of C'1 esterase activity.

The electrophoretic mobility of the protein associated with C'1 esterase activity was characterized by carrying out electrophoresis of C'1 esterase and normal human serum on the same glass plate. Immunoprecipitation lines of the separated proteins of the normal human serum were produced with antiserum. The zones of unlysed cells were compared directly with the immunoprecipitation lines (Fig. 4). Under these conditions, the C'1 esterase protein migrates in the zone between the α_1 and α_2 globulins.

DISCUSSION

A technique combining electrophoresis and immune haemolysis in a mixture of agarose and EA as the supporting medium, has been developed to detect C'1 esterase activity and to localize the protein associated with its activity. The method was applied to detect C'1 esterase activity in partially purified C'1 esterase preparations and in sera from affected members of a family with hereditary angioneurotic oedema.

The principle of the technique was based on the ability of C'1 esterase to inactivate C'4 and C'2 (Haines & Lepow 1964b; Donaldson and associates 1963, 1964) and according to Pondman & Peetoom (1964) also β_{1C}. After electrophoresis and application of C', the localized C'1 esterase destroys C' components and produces a zone of unlysed cells in the supporting medium. The absence of C'1 esterase in other areas of the gel allows the added C' to lyse EA in these areas. The zones of unlysed cells is indicative of C'1 esterase activity since the formation of zones could be overcome by inhibiting C'1 esterase activity with C'1 esterase inhibitor or by heating. This technique is more sensitive than the esterolytic technique for the demonstration of C'1 esterase activity. The electrophoretic mobility of the protein associated with C'1 esterase activity was established by direct comparison of zones of unlysed cells with immunoprecipitation lines formed with normal human serum and anti-total human serum. Under the condition used, the C'1 esterase protein migrates in the zone between α_1 and α_2 globulins. Haines & Lepow (1964a) first reported that the C'1 esterase protein migrated as an α_2-globulin in paper electrophoresis. Later, Haines & Lepow (1964c) investigated the C'1 esterase protein in immunoelectrophoresis and re-

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HYPERBARIC OXYGEN TREATMENT FOLLOWING INJECTION OF TETANUS TOXIN IN MICE

By

Johs Bo

Received 24 iv 65

Hyperbaric oxygen therapy has given promising results in the treatment of gas gangrene (*Boerema & Brummelkamp* 1960, *Smith et al* 1962 *Brummelkamp et al* 1963 *Boerema* 1964 *Wallyn et al* 1964) Recent reports indicate that such treatment may also be beneficial in clinical tetanus (*Lippincott & Harter* 1963 *Brummelkamp* 1964 *Pascale et al* 1964 *Winkel & Kroon* 1964) The few experimental studies available do not confirm this favourable impression (*Kelley & Pace* 1963 *Brummelkamp* 1964 *Fredette* 1964 *Galletta et al* 1964) although the theoretical background is sound as the growth of anaerobic micro organisms may be hindered by increasing the oxygen tension in the tissues and the tetanus toxin is reputedly oxygen sensitive

In view of the lack of correlation between clinical impression and experimental results an attempt was made to reassess the situation experimentally A pressure of 3 atmospheres absolute pressure was chosen as this is most commonly used in clinical work Pilot experiments were carried out to determine the exposure time the mice could tolerate at this pressure without obvious toxic effects

MATERIALS AND METHODS

Animals — Adult albino mice were used They were fed on commercial pellets and water

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MATERIALS AND METHODS

Animals —Adult albino mice were used They were fed on commercial pellets and water

Oxygen treatment —A cylindrical iron chamber was used 41 cm in length 15 cm in diameter volume 7¼ litres The removable front had a window which permitted [illegible] Ten mice were placed in a wire basket [illegible]

[illegible] toxin stored at 2 6° C [illegible] just before injection in a peptone phosphate buffer containing 0 75 per

cent peptone to stabilize the toxin in high dilutions The minimal lethal dose (MLD) (defined for this study as that amount which kills onehalf of a group of mice within 120 hours at room temperature) was about 1 35 $\times 10^{-6}$ ml per mg body weight Doses were adjusted according to weight of the animal, the volume of each dose being 0 4 0 5 ml The toxin was injected subcutaneously on the back of the animal

EXPERIMENTAL PROCEDURE

1 *The Effect of Oxygen under Increased Pressure Alone*

Male and female mice, weighing from 25 to 35 grams were subjected to oxygen at 3 atmospheres absolute pressure for various times ranging from ½ to 3 hours as shown in Table 1 The onset of signs of intoxication cleaning movements salivation gaping, jerky and deep respirations, restlessness terminating in convulsions was recorded The mice which died during a two week observation period were also recorded (Table 1)

TABLE 1

The Sex Difference in Response to Oxygen under 3 Atmospheres Absolute Pressure in Mice

Oxygen exposure (hrs)	Female Total No	Female Survived	Female Died	Male Total No	Male Survived	Male Died
½	5	5	0	5	5	0
1	5	5	0	5	5	0
1½	10	10	0	20	18	2
2	20	20	0	20	19	1
3	5	5	0	5	1	4
Total (%)	45	45 (100)	0 (0)	55	48 (87 3)	7 (12 7)

2 *Hyperbaric Oxygen Treatment Following Injection of Tetanus Toxin*

On the basis of the results of the previous experiment, female mice (weighing from 14 to 26 grams) were used as they were found to be more tolerant to oxygen treatment than males They were challenged to increasing doses of tetanus toxin The oxygen treatment was started 5 hours after toxin was given and lasted for one hour The survival time after toxin injection was recorded the mice being observed at intervals of 2 hours when they showed signs of approaching death

RESULTS

1 *The Effect of Oxygen under Increased Pressure Alone*

Signs of intoxication began after 1¼ hours exposure in most males and soon became pronounced In females only a few showed moderate signs of intoxication when 1¾ hours of exposure had passed This sex difference is also evident in Table 1 which shows that seven males and no female died out of a total of 110 mice

2 *Hyperbaric Oxygen Treatment Following Injection of Tetanus Toxin*

No animal demonstrated signs of tetanus intoxication at the time of oxygen treatment The mean survival times are recorded in Table 2 In

all groups the individual survival times showed a skewed distribution. When logarithmic ($\log_{10}$) transformation of the survival times was performed, an approximate normal distribution of the measurements was obtained. In the statistical evaluation of the results, therefore, the logarithmic values are used. The untreated controls showed an increase in mean survival time with decreasing dosage of toxin as expected. In the treated mice the mean survival time also increased with decreasing dosage. The mean survival time at 16 MLD was like that in the controls, but all lower dosages showed prolonged mean survival time after treatment. The progression in prolongation of mean survival time with decreasing dosage is marked. The scatter in all groups is, however, great. Hence the difference in mean survival time is statistically significant at 1.4 MLD only ($0.05 > P > 0.025$).

TABLE 2

Doses of realment

(10 ♀ mice in each group)

Dose of toxin	Mean survival time (hrs) Oxygen	Nil	Significance of difference between groups†
16 MLD†	54.1 ± 24.7	54.1 ± 22.9 *2	$P > 0.25$
12 MLD	54.9 ± 21.4	44.4 ± 10.6 *2	$P > 0.25$
2 MLD	72.7 ± 14.2	67.8 ± 13.1	$P > 0.25$
1.4 MLD	118.0 ± 17.1	97.6 + 23.6	$0.05 > P > 0.025$
1 MLD	230.4 ± 180.8	134.6 ± 37.5 *1	$0.10 > P > 0.05$

* Number of mice excluded due to technical error. † See text

DISCUSSION

The pilot experiments showed that female mice tolerated oxygen therapy at 3 atmospheres absolute pressure better than males. Therefore females were used in the main experiments with tetanus toxin. To avoid signs of oxygen intoxication, an exposure time of one hour was chosen.

The mice challenged to tetanus toxin showed a progressive beneficial effect of hyperbaric oxygen therapy as dosage of toxin decreased. Experiments with low dosages, however, were more difficult to assess. The problems concerned with the increasing scatter is clearly demonstrated by the 1 MLD group in the present investigation. If the experiment had been concluded after an arbitrary period of observation, *i.e.* 400 hours, the difference in the mean survival times in this group, as in the 1.4 MLD group, would have been statistically significant ($0.05 > P > 0.025$).

A possible favourable effect upon tetanus infection following hyperbaric oxygen therapy must be related to the increased oxygen tension

of the tissues. This increase may inhibit the growth of the anaerobic *Clostridium tetani* and thereby prevent toxin production. It may also enhance detoxification of the toxin.

Kelley et al. (1963) did not find any favourable effect of hyperpressure oxygen in mice inoculated with large doses of spores of *Clostridium tetani* in the crushed hamstrings. *Fredette* (1964) treated mice with oxygen at 3 atmospheres absolute pressure for 2 hours after toxin injection. He did not obtain any prolongation in the survival time. But he used doses of toxin which gave short survival times. Thus his experiment may be comparable with the 16 MLD group in the present experiment in which no prolongation in mean survival time was found. In addition his observations are widely spaced. Thus any slight beneficial effect might be lost. Experiments performed with exposure of the diluted, stabilized toxin to oxygen under increased pressure (*Fredette* 1964, *Galella et al.* 1964), do not reflect conditions as they are in the organism challenged to tetanus toxin. Pertinent conclusions can therefore scarcely be drawn from such experiments.

In clinical tetanus the most favourable results are obtained by *Pascale et al.* (1964) who experienced active regression of symptoms following hyperbaric oxygen treatment in their 9 treated cases. They, by degrees, developed sufficient confidence in the response of tetanus patients to this treatment that they at last used it almost exclusively in this disease. The active regression can imply an effect upon the toxin which the present investigation also suggests.

Repeated oxygen exposures in mice did not give better results in this investigation. Some of the observations lead to the impression that oxygen toxicity under these circumstances overweighed the beneficial effect on the tetanus toxin. When the favourable effect seems to be easier to demonstrate in clinical than in experimental tetanus, this may be due to the higher oxygen tolerance in man than in many experimental animals, including mice. The discrepancy between the beneficial clinical and largely negative experimental results following hyperbaric oxygen therapy, was also experienced with gas gangrene (*Brummelkamp et al.* 1963). It is possible that differences in oxygen sensitivity may explain these negative results too.

SUMMARY

Male mice showed less oxygen tolerance under 3 atmospheres absolute pressure than females.

Female mice injected with decreasing doses of tetanus toxin were treated for one hour in a hyperbaric chamber (3 atm. abs.) 5 hours after injection. The mean survival time was progressively prolonged with decreasing doses of tetanus toxin, but the difference between treated and control groups was statistically significant ($P < 0.05$) only at 1.4 MLD.

It is suggested that in mice the toxic effects of oxygen under pres
ure may overweigh the beneficial effects of treatment, in contrast to
e situation in man

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IMMUNOCHEMICAL STUDIES ON SOME SEROLOGICAL CROSS-REACTIONS IN THE *KLEBSIELLA* GROUP

14 *Oxidized Capsular Polysaccharides as Antigens*

By

JORUNN ERIKSEN

Received 25 ii 65

During the investigation of the structure of the capsular polysaccharides from *Klebsiella* type 3(C), *Klebsiella aerogenes* strain B 1076/48 and "Enterobacter" strain 349,(2,3) the polysaccharides were oxidized by periodate in order to give information about the linkages The oxidized polysaccharide was isolated and used for further chemical analysis, and as antigen to see if it still precipitated antibodies from homologous antiserum By using the oxidized and unoxidized capsular polysaccharide from *Klebsiella aerogenes*, strain B 1076 48, as antigens in a gel precipitation, two precipitation lines were observed which showed non-identity The gel precipitation can be seen in Fig 1 This indicated two different antibody-antigen systems The purpose of this paper will be a closer investigation of this observation

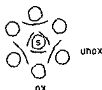
MATERIAL AND METHODS

Antigens

The capsular acidic polysaccharides from the following strains were used as antigens

Klebsiella pneumoniae	type 1 (A) strain 1265
Klebsiella pneumoniae	type 3 (C) strain F 10 N Y
Klebsiella pneumoniae	type 3 (C) strain 270/60
Klebsiella ozaenae	type 3 (C) strain 3828/60
Klebsiella rhinoscleromatis	type 3 (C) strain 92/04
Aerobacter (Klebs) aerogenes	type 3 (C) strain M A 73
Klebsiella ozaenae	type 4 (D) strain 4461/62
Klebsiella ozaenae	type 5 (E) strain 025

The capsular polysaccharides of the same strains as mentioned above were oxidized in the dark at room temperature by periodate as described earlier (2) For some strains *Klebsiella pneumoniae* type 1 (A) strain 1265 *Klebsiella ozaenae* type 4 (D) strain 4461/62 and *Klebsiella ozaenae* type 5 (E) strain 025 the consumption of periodate was determined (7) The oxidation of the polysaccharides was stopped by addition of ethylene glycol followed by dialysis *against running* tap water for 20 hr and isolation by *freeze* drying



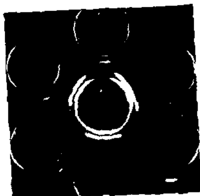


Fig 1

Antiserum against *Klebsiella aerogenes* strain B 1076/48 in the central well Oxidized and unoxidized capsular polysaccharides alternately as antigens in the peripheral wells

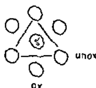




Fig 2

In the central well is antiserum against *Klebsiella pneumoniae* type 3 (C) strain F 10 N Y from which antibodies against capsular polysaccharide are removed Oxidized and unoxidized capsular polysaccharides alternately as antigens in the peripheral wells

Antisera

Rabbit antisera against all the strains mentioned above were prepared as described earlier (4) In addition, antisera against *Klebsiella* type 11, type 60 and type 21 were also used (6)

The quantitative precipitin determinations and the gel precipitations used as serological methods were the same as those used earlier (4)

RESULTS

The observation of a specific antibody against oxidized homologous capsular polysaccharide in anti *Klebsiella aerogenes* serum, strain B 1076/48, had to be confirmed Because of the similarity in the structure of the capsular polysaccharides of *Klebsiella aerogenes* strain B 1076/48 and *Klebsiella* type 3(C), five strains belonging to *Klebsiella*

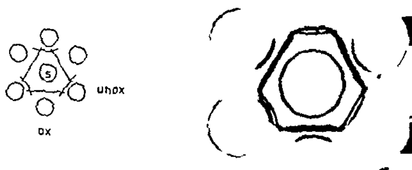


Fig 3
Antiserum against *Klebsiella pneumoniae* type 3 (C) strain F 10 N Y in the central well Oxidized and unoxidized capsular polysaccharides alternately as antigens in the peripheral wells

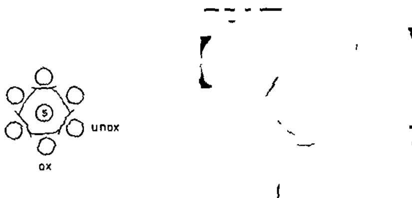


Fig 4
Antiserum against *Klebsiella ozaenae* type 3 (C) strain 3828/60 in the central well *Oxidized and unoxidized* capsular polysaccharides alternately as antigens in the peripheral wells

type 3(C) were investigated In antiserum against *Klebsiella rhinoscleromatis* type 3(C) strain 92/04, no specific antibody against the oxidized capsular polysaccharide could be seen by gel precipitation In the four other anti *Klebsiella* type 3(C) sera, the specific antibody against oxidized homologous capsular polysaccharides was observed Antisera against *Klebsiella pneumoniae* strain F 10 N Y and against *Klebsiella ozaenae* strain 3828 60 both type 3(C) seemed to contain most of this fraction This is demonstrated in Figs 2, 3 and 4

To be sure that the precipitation between the oxidized polysaccharide and its antibody was no unspecific reaction the oxidized polysaccharide from *Klebsiella pneumoniae* type 3(C) strain F 10 N Y was used as antigen for precipitation in five other antisera None of these sera showed any cross-reaction with *Klebsiella* type 3(C) The antisera used

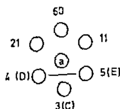




Fig 5
Oxidized capsular polysaccharide from *Klebsiella pneumoniae* type 3 (C) strain
60 *Klebsi*
ype 3 (C),
peripheral

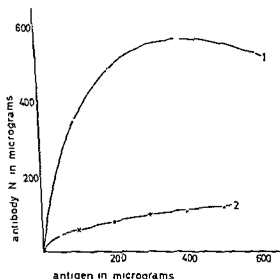


Fig 6
Quantitative precipitin determination in antiserum against *Klebsiella pneumoniae* type 3 (C) strain F 10 N Y Curves show µg antibody N per ml serum Curve 1 Capsular polysaccharide as antigen Curve 2 Oxidized capsular polysaccharide as antigen

were against *Klebsiella ozaenae* type 5(E) strain 025, *Klebsiella ozaenae* type 4(D) strain 03, *Klebsiella* type 11, type 21 and type 60 The result of this gel ... from Fig 5 There was r ... 3(C) polysaccharide and a

A quantitative precipitin determination was then carried out in antiserum against *Klebsiella pneumoniae* type 3(C) strain F 10 NY. Oxidized and unoxidized homologous capsular polysaccharides were used as antigens. The results can be seen from Fig. 6. It seemed to have no influence on the quantity of antibodies precipitated by the oxidized polysaccharide, whether the type specific antibodies had been removed from antiserum or not.

The presence of antibody against oxidized capsular polysaccharide in antiserum from rabbits immunized with *Klebsiella* type 3(C) has been demonstrated. The same occurrence has to be investigated in other types of *Klebsiella*.

The capsular polysaccharide of *Klebsiella ozaenae* type 4(D) has been investigated earlier (8). By isolation and further purification of the capsular polysaccharide from *Klebsiella ozaenae* type 4(D) strain 4461/62 by means of cetyl pyridinium chloride, a product was obtained showing $[\alpha]^{20}_{D} = +102.5°$ (c 0.3 water). By hydrolysis and paper chromatography it showed to contain uronic acid, glucose and mannose. This polysaccharide was used as antigen.

The same polysaccharide was oxidized by periodate and isolated after 5 hr and 72 hr to be used as antigen. The number of moles of sodium periodate consumed per mol anhydrosugar was 5 hr 0.69, 24 hr 1.06, 48 hr 1.23 and 72 hr 1.35. The oxidized polysaccharide isolated after 72 hr, showed by paper chromatography a decrease in the content of glucose.

In anti *Klebsiella ozaenae* type 4(D) serum, no antibody against oxidized homologous polysaccharide could be demonstrated, either after oxidation for 5 hr or for 72 hr.

The same experiment was carried out with *Klebsiella ozaenae* type 5(E). The capsular polysaccharide from *Klebsiella ozaenae* type 5(E) strain 025 has also been investigated earlier (5). By further purification of the same polysaccharide a fraction was obtained $[\alpha]^{20}_{D} = -32.6°$ (c 0.3 water) which by paper chromatography showed to contain glucose, mannose and uronic acid. This polysaccharide was used as antigen. By oxidation of the same polysaccharide the number of moles sodium periodate per mol anhydrosugar was 5 hr 0.27, 24 hr 0.38, 48 hr 0.49 and 72 hr 0.61. The oxidized polysaccharide from *Klebsiella* type 5(E) was isolated after 5 hr and 72 hr to be used as antigen. Paper chromatography of the polysaccharide, oxidized for 72 hr showed a decrease in the content of uronic acid.

In antiserum against *Klebsiella ozaenae* type 5(E) no specific antibody against oxidized capsular polysaccharide could be seen. Whether the polysaccharide was oxidized for 5 hr or for 72 hr, no difference could be observed.

Capsular polysaccharides, isolated from *Klebsiella ozaenae* type 4(D) and type 5(E) consumed more periodate by oxidation than did the capsular polysaccharide of *Klebsiella type* 3(C). This only consumed

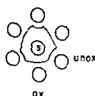




Fig 7

Antiserum against *Klebsiella pneumoniae* type 1 (A) strain 1264 in the central well Oxidized and unoxidized capsular polysaccharides alternately as antigens in the peripheral wells

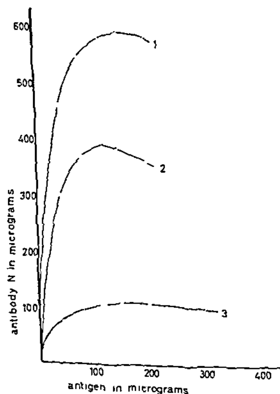


Fig 8

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pneumoniae
e 1 Capsular
e as antigen
from which

A quantitative precipitin determination was then carried out in anti serum against *Klebsiella pneumoniae* type 3(C) strain F 10 NY Oxidized and unoxidized homologous capsular polysaccharides were used as antigens The results can be seen from Fig 6 It seemed to have no influence on the quantity of antibodies precipitated by the oxidized polysaccharide, whether the type specific antibodies had been removed from antiserum or not

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The capsular polysaccharide of *Klebsiella ozaenae* type 4(D) has been investigated earlier (8) By isolation and further purification of the capsular polysaccharide from *Klebsiella ozaenae* type 4(D) strain 4461/62 by means of cetyl pyridinium chloride, a product was obtained showing $[\alpha]_D^{20} = +102.5°$ (c 0 3 water) By hydrolysis and paper chromatography it showed to contain uronic acid, glucose and mannose This polysaccharide was used as antigen

The same polysaccharide was oxidized by periodate and isolated after 5 hr and 72 hr to be used as antigen The number of moles of sodium periodate consumed per mol anhydrosugar was 5 hr 0 69, 24 hr 1 06, 48 hr 1 23 and 72 hr 1 35 The oxidized polysaccharide isolated after 72 hr, showed by paper chromatography a decrease in the content of glucose

In anti *Klebsiella ozaenae* type 4(D) serum, no antibody against oxidized homologous polysaccharide could be demonstrated, either after oxidation for 5 hr or for 72 hr

The same experiment was carried out with *Klebsiella ozaenae* type 5(E) The capsular polysaccharide from *Klebsiella ozaenae* type 5(E) strain 025 has also been investigated earlier (5) By further purification of the same polysaccharide a fraction was obtained $[\alpha]_D^{20} = -32.6°$ (c 0 3 water) which by paper chromatography showed to contain glucose, mannose and uronic acid This polysaccharide was used as antigen By oxidation of the same polysaccharide the number of moles sodium periodate per mol anhydrosugar was 5hr 0 27, 24 hr 0 38, 48 hr 0 49 and 72 hr 0 61 The oxidized polysaccharide from *Klebsiella* type 5(E) was isolated after 5 hr and 72 hr to be used as antigen Paper chromatography of the polysaccharide, oxidized for 72 hr, showed a decrease in the content of uronic acid

In antiserum against *Klebsiella ozaenae* type 5(E) no specific antibody against oxidized capsular polysaccharide could be seen Whether the polysaccharide was oxidized for 5 hr or for 72 hr, no difference could be observed

Capsular polysaccharides isolated from *Klebsiella ozaenae* type 4(D) and type 5(E) consumed more periodate by oxidation than did the capsular polysaccharide of *Klebsiella type* 3(C) This only consumed

from *Klebsiella pneumoniae* type 1(A) is suggested to be linked 1 3 the periodate would only attack the terminal end groups The importance for the specificity of terminal groups in the polysaccharide is therefore clearly demonstrated by this cross reaction However, when the antibodies against the homologous polysaccharide were removed the serum still contained a specific antibody against the oxidized polysaccharide

Both from Fig 6 and Fig 8 it will be noted that a large quantity of oxidized polysaccharide is required in order to precipitate a small quantity of antibody This indicates a cross reaction suggesting the possibility that the antigen causing the appearance of these antibodies is similar to but not identical with the oxidized polysaccharide

Another interesting point is that in antisera where a specific antibody against the oxidized polysaccharide was observed the type specific capsular polysaccharide only consumed a small quantity of periodate by oxidation The polysaccharide from *Klebsiella pneumoniae* type 1(A) consumed no measurable quantity of periodate and the polysaccharide from *Klebsiella* type 3(C) consumed only 0 33 mol periodate per mol anhydrosugar by oxidation for 72 hr Both these oxidized polysaccharides precipitated a specific antibody from homologous antisera

On the other hand the polysaccharides from *Klebsiella ozaenae* type 5(E) and type 4(D) consumed respectively 0 61 and 1 35 mol periodate per mol anhydrosugar by oxidation in 72 hr These oxidized polysaccharides did not precipitate any specific antibody from homologous antisera

The rabbits were injected with the whole bacteria and would have formed antibodies against all antigens present one of which is the type specific acidic polysaccharide Since the rabbits also produce antibodies which react with the oxidized but not with the unoxidized polysaccharide it is tempting to propose the hypothesis that an oxidation of the capsular antigen takes place *in vivo*, which leads to the production of an antigen with a structure closely related to that of the periodate oxidized polysaccharide If the capsular polysaccharide like other polysaccharides behaves as hapten in rabbits the oxidation *in vivo* would have to be assumed to occur while the acidic polysaccharide is still combined with such other components of the bacteria as would be needed to make it a complete antigen

The observations reported in this paper indicate that a polysaccharide antigen may induce production of antibodies not only against the *polysaccharide itself* but also against decomposition products of different antigenic specificities formed *in vivo* Thus not all antibodies produced in response to the introduction of an antigen must necessarily react with the *antigen* It seems that studies of the reactions of such immune sera with chemical modifications of the antigen might give clues to the manner of breakdown of the antigen *in vivo* Oxidation might be one way of decomposing the capsular antigen

0 33 mol periodate per mol anhydrosugar (2) Another capsular polysaccharide, isolated from *Klebsiella pneumoniae* type 1(A) strain 1265 consumed no measurable periodate by oxidation This polysaccharide has recently been closely investigated (1) and was suggested to be linked 1,3

Capsular polysaccharide from *Klebsiella pneumoniae* type 1(A) strain 1265 was used as antigen The polysaccharide was also oxidized The failure of consumption of measurable quantities of periodate was confirmed The oxidized polysaccharide was isolated, to be used as antigen too

Antiserum against *Klebsiella pneumoniae* type 1(A) strain 1265 contained a specific antibody against oxidized homologous polysaccharide This is demonstrated in Fig 7 A quantitative precipitin determination was carried out in anti *Klebsiella pneumoniae* type 1(A) strain 1265 serum which can be seen from Fig 8 When oxidized homologous polysaccharide was used as antigen, 65 per cent of the antibodies was precipitated However, when antibodies against the capsular polysaccharide were removed beforehand, and the supernatant was precipitated with oxidized capsular polysaccharide, still 18 5 per cent of the antibodies was precipitated This must be due to the content of specific antibodies against the oxidized polysaccharide

DISCUSSION

In antisera against *Klebsiella aerogenes*, strain B 1076 48, *Klebsiella* type 3(C) and *Klebsiella pneumoniae* type 1(A) strain 1265, a specific antibody against the homologous periodate-oxidized capsular polysaccharide was observed In antisera against *Klebsiella ozaenae* type 4(D) and 5(E) this antibody could not be demonstrated

Fig 6 gives the results of the quantitative precipitation in anti *Klebsiella* type 3(C) serum, with homologous capsular polysaccharide, before and after oxidation, as antigens Both polysaccharides precipitated antibody from the immune serum The most obvious explanation of this reaction might be that the oxidized polysaccharide retained sufficient structural similarity to the unoxidized polysaccharide to cross-react with some of the antibody against the latter But when the antibodies against unoxidized polysaccharide were removed from the immune serum, the oxidized polysaccharide still precipitated the same quantity of antibodies This reaction confirms the presence of a specific antibody directed *only against the oxidized polysaccharide*

In Fig 8 the precipitation with oxidized and unoxidized homologous polysaccharide as antigens in anti *Klebsiella pneumoniae* type 1(A) serum is demonstrated The oxidized polysaccharide precipitates 64 per cent of the quantity of antibodies precipitated with unoxidized polysaccharide This could be a cross reaction due to structural similarities between the polysaccharides concerned Since polysaccharide

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STUDIES ON TRANSFORMATION IN *MORAXELLA* AND ORGANISMS ASSUMED TO BE RELATED TO *MORAXELLA*

5 *Streptomycin resistance transformation between serum-liquefying, nonhaemolytic moraxellae,* Moraxella bovis *and* Moraxella nonliquefaciens

By

KJELL BØVRE

Received 14 V 65

A single strain of *Moraxella liquefaciens* (strain 7911 of the present material) has been employed in preliminary transformation studies by *Bøvre & Henriksen* (1962) and by *Catlin & Cunningham* (1964) Neither group of investigators claimed to have elicited significant transformation of or by this strain However, *Bøvre* (1963) reported low-frequency transformation of Neisseria catarrhalis with DNA extracted from a strain of *Moraxella liquefaciens* These are the *only* preexisting communications on transformation involving serum liquefying, nonhaemolytic moraxellae

In line with previous investigations of this series, the present report concerns the examination of several strains with the mentioned characteristics, and their relations to representative strains of *Moraxella nonliquefaciens* and *Moraxella bovis* (*Bøvre* 1964b, 1965a) The relations to the strains 19116 51 and 752/52, both found to deviate in terms of streptomycin resistance transformation from *Moraxella nonliquefaciens* and *Moraxella bovis* (loc. cit), are also noted

MATERIAL AND METHODS

Strains with the [illegible]

SUMMARY

Oxidized capsular polysaccharides from different types of *Klebsiella* as antigens have been serologically investigated.

Antibody which reacted with oxidized, but not with unoxidized type specific polysaccharide, was demonstrated in anti *Klebsiella pneumoniae* type 1(A) and anti *Klebsiella* type 3(C) sera from rabbits.

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Transformants were counted in 0 1 ml quantities of proper dilutions of the transformation mixture Ratios of inter to intrastrain transformation were only calculated from transformant counts obtained in simultaneous transformation of aliquots of one and the same recipient population by the different DNAs in question Differential dilution was undertaken *after* termination of DNA absorption by means of DNase

Spontaneous streptomycin resistant mutants were used as donors when ratios of inter to intrastrain transformation frequencies were to be determined This has been the general rule all from the beginning of the author s studies on transformation in *Moraxella* and *Neisseria* The only exception so far has been one *Moraxella nonliquefaciens* donor a streptomycin resistant transformant of strain 159/62 which has not been employed in assumed interspecies reactions (*Bovre* 1964 b) However transformant DNA was used as before (*Bovre* 1965 a b) in experiments designed for the elucidation of homology or heterology of the streptomycin resistance determinant itself

Continuous DNA exposure (without DNase termination of DNA absorption before plating) was used in initial experiments with strains of low transformability This semi quantitative variety of the procedure has been characterized previously (*Bovre* 1964 b 1965 b)

As before some questions of a methodological nature are discussed in the section of results

RESULTS

Description of the Serum Liquefying, Nonhaemolytic Strains

Microscopical examination revealed Gram negative, thick rods of varying length Some strains presented long filaments among normally dividing organisms particularly pronounced in strain 9985 (Fig 1) The strains 9833 and A947(1) had the microscopical appearance of *Moraxella nonliquefaciens* 836 61 and the strains 7911, 11748, E 1 and L1 that of *Moraxella nonliquefaciens* 4663 62 (*Bovre* 1964b, Figs 1 and 2) However, the strains E1 and L1 generally had a slightly smaller cell diameter than the other strains Variation of plumpness was often evident in one and the same preparation Some resistance to decolorization during Gram staining was observed in all strains The appearance was approximately the same after incubation at 37° C, as compared with 32 33° C



Fig 1

Moraxella liquefaciens 998 stained with Löffler s methylene blue × 960

London, and the strains L1 and I1 from Dr W J Ryan The latter two strains were guinea pig isolates

The above mentioned strains were examined according to the same conventional methods and with the same media as described for *Moraxella nonliquefaciens* and *Moraxella bovis* (Bøvre 1964 b, 1965 a) As described for the examination of *Neisseria* (Bøvre 1965 b), a set of improved serum media for indol, nitrite and hydrogen sulphide production was employed parallel to the original simple media in question

The assessment of fastidiousness was extended and included tests for growth on the following media

Hugh & Leifson's solid peptone medium (prepared with Bacto Peptone and glucose)—pH 7 1

Koser's citrate medium—pH 6 8

Koser's citrate medium with 1 5 per cent agar

Dextrose Broth (Difco)—pH 7 2

Dextrose Broth (Difco) with 1 5 per cent agar

Brain Heart Infusion (Difco)—pH 7 4

Brain Heart Infusion (Difco) with 1 5 per cent agar

1 per cent Proteose Peptone no 3 (Difco) and 0 5 per cent NaCl in distilled water —pH 7 6 (ordinary 1 per cent peptone water)

Three media consisting of 1 5 per cent agar in peptone water (with 0 5 per cent NaCl and 1 per cent of Proteose Peptone no 3 (Difco) Bacto Peptone and Pepton aus Casein, tryptisch verdaut (Merck), respectively)—pH 7 6

Growth on the media listed was tested with small inocula and subcultures on the same medium

Generally, tests were performed at 32 33° C and at 37° C Growth in ordinary 1 per cent peptone water was tested only at the former incubation temperature

The strains 7911, 9833, 9985 L1 and I1 were tested for antibiotic sensitivity as described (Bøvre 1964 b) In sensitivity tests with the strains A947(1) and 11748 approximate minimum inhibitory concentrations were not calculated because of slow growth on the standard medium With the latter strains zones of growth inhibition were measured on chocolate ascites agar (8 5 per cent heated horse blood and 15 per cent human ascites in 1 5 per cent nutrient agar—pH 7 4) The latter medium was also used for the maintenance of the two strains by subculture every third day for long periods

Moraxella nonliquefaciens *Moraxella bovis* and the deviating strains 19116/51 and 752/52 have been examined previously All strains of these groups (Bøvre 1964 b 1965 a) also those not included in the present transformation experiments were retested for indol nitrite and hydrogen sulphide production with serum enriched media (Bøvre 1956 b) Notes have been made in previous communications on growth in the simple original media for these purposes only to describe the conditions under which the biochemical tests were performed Small inocula were not used in these tests For comparison with the serum liquefying nonhaemolytic strains *Moraxella nonliquefaciens* and *Moraxella bovis* were subjected to tests for fastidiousness beyond those previously employed for these bacteria

The methods of mutant selection DNA extraction and quantitative streptomycin resistance transformation were in accordance with the original description (Bøvre 1964 a) and experience obtained with other assumed species and genera (Bøvre 1964 b, 1965 a b) Chocolate ascites agar was however used instead of human blood agar for the selection of transformants of strain A947(1) The procedure included selection of transformants at different concentrations of streptomycin control of donors and transformants by means of velvet replica plating control of the effectiveness of DNase termination of DNA absorption control for mutation simulating transformation and control of DNA saturation at the different recipient densities The DNA extracts were adjusted to 200 μg per ml roughly estimated with the Dische diphenylamine reaction and used in 0 1 or 0 2 ml quantities per ml of the recipient/DNA mixture during DNA absorption

The results of *Catlin & Cunningham* (1964) indicate that strain 19116/51 in particular is sensitive to higher temperatures in transformation experiments The temperature 32 33° C, which has been used in all previous studies of the present series, was maintained also for the incubation of transformants before assay There is no indication that this relatively low temperature is deleterious to transformant survival and growth (to be published) In many instances the incubation period was prolonged beyond the standard 3 days in some cases for as long as 7 days

was growth on glucose ascites agar and on inspissated bovine serum (a little inferior growth of the strains A947(1) and 11748 on the latter medium).

The strains 7911, 9833, 9985, E1 and L1 grew fairly well in Dextrose Broth and in Brain Heart Infusion. Some irregularities were observed in the fluid media. Thus, the strain 9833 on one occasion revealed visible turbidity in both media at 32–33° C, whereas neither medium supported growth at 37° C. On the surface of the corresponding solid media, however, growth was luxuriant of all 5 strains, at both temperatures. On the surface of solid 1 per cent peptone media, the 5 strains grew feebly to moderately with all kinds of peptone at the two temperatures. In ordinary 1 per cent peptone water, moderate inocula gave fairly good growth of the 5 strains.

The strains A947(1) and 11748 did not grow in Dextrose Broth at any temperature. In Brain Heart Infusion both strains grew feebly at 32–33° C, but strain 11748 showed no turbidity in a parallel test at 37° C. On the surface of these two media with agar added, the two strains grew with evenly distributed pin point colonies at both temperatures. On the solid peptone media strain A947(1) did not grow, whereas scanty pin point growth of strain 11748 occurred on the media with Proteose Peptone no 3 and with Pepton aus Casein at 32–33° C (not at 37°). No growth was seen on the medium with Bacto Peptone. Ordinary 1 per cent peptone water did not support growth of the two strains.

Among the strains which were excluded from the studies because streptomycin resistant mutants were lacking, two strains had the designation *Moraxella lacunata*. Both strains grew like the strains A947(1) and 11748 on the surface of Dextrose Broth and Brain Heart Infusion with agar added (no growth in the fluid media). Thus all available strains named *Moraxella lacunata* were able to grow on these solid meat extractive media, without the addition of serum or ascites.

The strains were all immotile, oxidase positive and did not produce acid from glucose (tested on glucose ascites agar and on Hugh & Leifson's medium). There was no production of indol or hydrogen sulphide. Other characteristics are presented in Table 1. (The tabulated results were identical at 32–33° C and 37° C. Nitrite production was observed in the serum-enriched medium as well as in the simple nitrate medium when the latter was inoculated heavily.) All strains were strongly sensitive to common antibiotics (Table 2).

Additional Description of Moraxella nonliquefaciens *and* Moraxella bovis

Wh[illegible] ciens (20
strai[illegible] 1911651
and [illegible] enriched media for indol, nitrite and hydrogen

The diameter of colonies on blood agar after incubation for 20 h at 32–33° C, was approximately 1 mm for the strains E1 and L1, and 1.5–2 mm for the strains 7911, 9833 and 9985. On this medium the colonies of the strains A947(1) and 11748 were of pin point size. On chocolate ascites agar, growth of the latter two strains was considerably improved. This improvement, however, was not sufficient to give 1 mm colony diameters in 20 h. The colonies were round, even, glistening and generally of the low conical type. Their opacity was not far from that of most *Moraxella nonliquefaciens* strains, but slightly more pronounced for the "large" colony strains. There was no pigmentation. *The consistency* of colonies varied somewhat (see Table I), most strains being soft, emulsifiable in physiological saline.

TABLE I
Some Characteristics of the Serum Liquefying Nonhaemolytic Moraxella Strains

Strain	Consistency of colonies	Agglutinability in physiological saline	Growth on the surface of Hugh & Leifson's medium	Growth in citrate[1]	Nitrite production	Urease activity	Haemolysis	Serum liquefaction
7911	Claylike	+	+†		+	—	—	++*
9833	Soft	—	+	—	+			+°
9985	Soft	—	+		+	—		++
E1	Almost soft	—	+	—	+	—	—	++
L1	Intermediate§	(+)	+		+	—		++
A947(1)	Soft			—	+	—	—	++
11748	Soft	—	—	—	+	—		++

[1] Tested in Koser's fluid medium and on the same medium with agar added. § Intermediate between soft and friable. † + = Feeble growth. * ++ = Rapid reaction clearly evident in 20 h. ° + = Relatively slow reaction evident in 2 days.

Growth on the surface of blood agar was not significantly influenced when the incubation temperature was raised from 32–33° C to 37° C. All strains also grew at 25° C. At this temperature, however, the strains A947(1) and 11748 needed incubation for 2 days to give visible colonies, even on chocolate ascites agar. The other strains were less influenced by the low temperature. No need for a humid growth atmosphere was observed. No strain was able to grow anaerobically (hydrogen atmosphere). In 0.4 per cent Brain Heart Infusion agar stab culture the 5 least fastidious strains grew fairly well down to 5 mm below the surface, as did the strains A947(1) and 11748 when the medium was enriched with 10 per cent horse serum. Growth in the original, simple media for indol, nitrite and hydrogen sulphide production was somewhat irregular (not observable with the strains A947(1) and 11748). In the improved serum media growth was good, and so

although all streptomycin resistant mutants used as donors in these and other experiments had been selected at 500 µg streptomycin per ml. Further, all donors possessed uniform resistance to 1000 µg streptomycin per ml and were streptomycin independent, as shown by means of replica plating of diluted surface cultures.

TABLE 3

Transformation between Serum Liquefying Nonhaemolytic Moraxella Strains. The Influence of Selection Concentration of Streptomycin on Transformant Counts in Various Recipient/Donor Combinations

Experiment no	Recipient strain	Donor strain	Number of transformants per plate at µg per ml of streptomycin 500	100	50	10
1	9833	9833	323	330	392	345
		7911	0	179	330	379
		9985	325	368	320	331
		F1	48	64	56	53
		L1	0	27	55	57
		A947(1)	150	123	182	151
		11748	256	207	223	242
2	F1	F1	298	352	320	300
		L1	57	131	316	305
		9833	120			106
		A947(1)	52		63	
3	A947(1)	A947(1)	281,306		290,311	277,322
		11748	279,282		265,280	276,335
		7911	0,0		54,66	69,74
		9833	60,67		52,72	55,64
		F1	*29,36*		32,36	20,28
		L1	*0,0*		*35,40*	*29,30*

Three experiments, each with simultaneous observations. *Experiments no 1 and 2* are semiquantitative (no termination of DNA absorption with DNase). Experiment no 3 is quantitative and basis for Table 4.

All transformant colonies selected at 10 µg streptomycin per ml in experiment no 1 of Table 3 were replicated to 50, 500 and 1000 µg streptomycin per ml. The transformants produced with the donors 9833, 9985, F1, *A947(1) and 11748* were without exception able to grow at 500 µg streptomycin per ml. No transformant obtained with the *donor* strain 7911 and only 2 of those produced by the donor strain L1 grew when *replicated to this* concentration of streptomycin. All transformants growing at 500 µg per ml, also grew at 1000 µg per ml. At 50 µg streptomycin per ml *all transformants* grew freely, as they did in replicas to media without streptomycin.

The above mentioned observations show that in these bacteria, contrary to earlier experience with *Moraxella nonliquefaciens* and *Moraxella bovis*, the selection concentration 500 µg streptomycin per *ml is* critical for transformant growth with some donors. Early unsuccessful

sulphide production and with the solidified Koser's citrate medium no result differed from that observed with the original medium in question (*Bovre* 1964 b, 1965 a). The results were the same at 32–33° C and 37° C, as was the outcome of the test for growth on the surface of Hugh & Leifson's medium, which was repeated at the two temperatures.

TABLE 2
Antibiotic Sensitivity of the Serum-Liquefying Nonhaemolytic Moraxella Strains. Ranges of Inhibition Zone Diameters and Approximate Minimum Inhibitory Concentrations (m.i.c.) [1]

Antibiotic	Strains 7911, 9833 and 9985		Strains E1 and L1		Strains A947(1) and 11748
	Zones in mm	M.i.c.	Zones in mm	M.i.c.	Zones in mm§
Penicillin	38–41	0.02–0.007	33–35	0.07–0.04	37–38
Streptomycin	28–30	0.1–0.05	25–26	0.4–0.3	25–26
Chloramphenicol	36–38	0.1–0.09	32–34	0.4–0.2	34–35
Oxytetracycline	30–33	0.08–0.03	26–28	0.3–0.2	30
Erythromycin	30–38	0.3–0.04	26	1	38

1 Method of *Ericsson, Hogman & Wickman* (1954). M.i.c. values calculated from zone diameters by means of regression equations for each antibiotic (*Ericsson* 1960). M.i.c. given as I.U./ml for penicillin, as µg/ml for the other antibiotics.
§ Examined on chocolate ascites agar.

Moraxella nonliquefaciens as a rule did not grow on the solid medium with Proteose Peptone no. 3, whereas *Moraxella bovis* grew rather well. On the other solid 1 per cent peptone media, all strains revealed feeble to moderate growth at the two temperatures. In ordinary 1 per cent peptone water, selected strains of *Moraxella nonliquefaciens* did not grow, contrary to *Moraxella bovis*.

Transformation Reactions

Because of low transformability, none of the less fastidious serum-liquefying, nonhaemolytic strains (7911, 9833, 9985, E1 and L1) could be used as recipient in quantitative transformation.

The transformability of the strains 9833 and E1, however, permitted their use as recipients in initial semiquantitative transformation with closely related donors. In Table 3 these experiments are presented together with quantitative transformation of the sufficiently transformable strain A947(1). Transformants primarily resistant to 500 µg streptomycin per ml are not encountered with the donors 7911 and L1,

with the donor *Moraxella bovis* is 1.2×10^{-3}, and the ratios observed with the donor *Moraxella nonliquefaciens* 4663/62 are ranging from 4.5×10^{-3} to 4.8×10^{-3}. The strain A947(1) is not transformed with DNAs from the strains 19116/51 and 752/52, as one would expect from previous negative reactions between the latter strains and *Moraxella bovis*/*Moraxella nonliquefaciens* (*Bøvre* 1964 b, 1965 a).

TABLE 5

Transformation of Strain A947(1) with DNAs from Moraxella bovis, Moraxella nonliquefaciens and the two Deviating Strains 19116/51 and 752/52

Donor strain	Recipient count/ml	Interstrain transformants/ml	Intrastrain transformants/ml‡	Ratio of inter- to intrastrain transformation
M. bovis 10900	4.0×10^8	7.6×10^2 (76)§	6.1×10^5 (61)	1.2×10^{-3}
M. bovis 10900	5.1×10^8	4.7×10^2 (47)	3.9×10^5 (385)	1.2×10^{-3}
M. nonliq. 4663/62	4.0×10^8	2.9×10^3 (294)	6.1×10^5 (61)	4.8×10^{-3}
M. nonliq. 4663/62	5.1×10^8	1.8×10^3 (175)	3.9×10^5 (385)	4.5×10^{-3}
Strain 19116/51	4.0×10^8	$< 10^1$ (0)†	6.1×10^5 (61)	$< 1.6 \times 10^{-5}$
Strain 752/52	5.1×10^8	$< 10^1$ (0)*	3.9×10^5 (385)	$< 2.6 \times 10^{-5}$

Duration of DNA exposure 15 min. Selection of transformants at 50 µg streptomycin per ml, in several instances with parallels at 500 and 10 µg per ml. ‡ Intrastrain transformants have been scored in simultaneous transformation of the recipient with its own mutant DNA. Identical intrastrain transformant counts indicate parallel experiments. § Figures in brackets indicate means of 3–6 plate counts (see text). † In parallel continuous DNA exposure 3 resistant colonies were detected per plate. * In parallel continuous DNA exposure transformants were still not detected.

Strain 19116/51 was also used as recipient in quantitative transformation with strain 9833 as the donor and selection concentrations of streptomycin down to 10 µg per ml. In this experiment the inter- to intrastrain transformation ratio was found to be less than 2.0×10^{-4} (not tabulated). In continuous DNA exposure, parallel to the latter experiment, only a couple of streptomycin resistant colonies occurred per plate. Like the results of continuous DNA exposure given in connection with Table 5, this experiment shows that the relations in terms of streptomycin resistance transformation between strain 19116/51 and the serum liquefying nonhaemolytic moraxellae are very distant as they are between this strain and *Moraxella nonliquefaciens*/*Moraxella bovis*.

In Table 6 is presented quantitative streptomycin resistance transformation of representative strains of *Moraxella nonliquefaciens* and *Moraxella bovis* by donors from the serum liquefying nonhaemolytic group of strains. No recipient seems to distinguish between the various heterologous DNAs, as the ratios of inter- to intrastrain transformation of one and the same recipient deviate very little from each other. The ratios observed with the recipient *Moraxella nonliquefaciens* 7784 are ranging from 1.0×10^{-3} to 1.4×10^{-3}, with the recipient *Moraxella non-*

attempts at transformation of a potent *Moraxella nonliquefaciens* recipient with *Moraxella liquefaciens* 7911 DNA (*Bøvre & Henriksen* 1962) were due low streptomycin resistance of transformants as compared with that of the donor. This was soon confirmed in experiments with reduced transformant selection concentration of streptomycin (not published).

Genetic analysis of the marker peculiarities of the strains 7911 and L1 might clarify whether the donors in question owe their high streptomycin resistance to a combination of individual low resistance mutations or to *modifier genes enhancing a low-resistance mutation* (*Bryan* 1961, *Rotheim & Ravin* 1961). It is of great importance to note that when the selection concentration is sufficiently lowered, the transformant yield is practically identical with that induced by presumptively closely related donors with "normal" markers (Table 3). Although the involved donors 7911 and L1 have been replaced by other donors in subsequent work, the consequence of these observations has been the application of 50 μg streptomycin per ml as the main selection concentration, with parallels at 500 and sometimes at 10 μg streptomycin per ml.

TABLE 4

Streptomycin Resistance Transformation Among Serum Liquefying Nonhaemolytic Moraxella *Strains. Ratios of Inter- to Intrastrain Transformation Frequency. Recipient Strain A947(1). Recipient Count 1.8 × 10^7 per ml*

Donor strain	Transformants/ml	Ratio of inter- to intrastrain *transformation*
A947(1)	3.0×10^{3} (298)*	
11748	2.9×10^{3} (286)*	9.6×10^{-1}
7911	6.6×10^{2} (66)§	2.2×10^{-1}
9833	6.2×10 (62)*	2.1×10^{-1}
I1	3.0×10 (30)*	1.0×10^{-1}
L1	3.4×10 (34)§	1.1×10^{-1}

Identical with experiment no. 3 of Table 3. Duration of DNA exposure 15 min. Selection concentrations 500, 50 and 10 μg streptomycin per ml. * Figure in brackets: Mean of 6 plate counts (all selection concentrations). § Figure in brackets: Mean of 4 plate counts (plates with 500 μg streptomycin per ml excluded).

The ratios of inter- to intrastrain transformation between 6 of the serum-liquefying, nonhaemolytic strains are shown in Table 4 to be situated between 9.6×10^{-1} and 1.0×10^{-1}. The semiquantitative results of Table 3 do not indicate that other recipient/donor combinations among these strains would have revealed much less compatibility in terms of quantitative streptomycin resistance transformation than expressed by the ratio 1.0×10^{-1}.

The strain A974(1) was transformed to streptomycin resistance with DNA extracts from representative *Moraxella bovis* and *Moraxella nonliquefaciens* strains. The data of these quantitative experiments are presented in Table 5. The ratio of inter- to intrastrain transformation

tration, as regards observations with means within the range 20–250 (*Bovre* 1964a) In one of the experiments with the recipient strain 7784 (Table 6), the counts at the selection concentration 500 µg per ml were systematically low and therefore excluded from the calculation of means In this case the low counts were considered due to a critically short phenotypic expression period as compared with plates of lower streptomycin concentrations (inhibiting concentration of streptomycin being obtained earlier in the growth zone as the amount of diffusing streptomycin increases)

Replicas of transformants from all recipient/donor combinations in Tables 5 and 6 revealed uniform resistance to 1000 µg streptomycin per ml The only difference found between recipients and corresponding transformants in simple tests, was the acquired streptomycin resistance of the latter

TABLE 7

Effect on Transformation Frequency of the Integration of Heterologous Streptomycin Resistance Marker in Donor DNA

Recipient strain	Recipient count/ml	Donor DNA	Transformants/ml
Strain A947(1)	5 1 10^8	(A947(1)Sm^r)	3 9 10^5
		(A947(1)Sm^r4663/62)	3 8 10^5
		(A947(1)Sm^r10900)	3 9 10^5
Moraxella bovis 10900	5 0 10[illegible]	(10900Sm^r)	9 1 10[illegible]
		(10900Sm^rA947(1))	8 8 10[illegible]
Strain A947(1)	4 0 10^8	(10900Sm^r10900)	8 1 10
		(10900Sm^rA947(1))	3 0 10^3
Moraxella bovis 10900	1 2 10^8	(A947(1)Sm^r)	2 0 10
		(A947(1)Sm^r10900)	9 7 10[illegible]
Moraxella n nliquefaciens 4663 62	9 4 10^7	(A947(1)Sm^r)	6 8 10^2
		(A947(1)Sm^r4663/62)	4 8 10^3

(A947(1)Sm^r) and (10900 Sm^r) = DNAs of spontaneous mutants (10900Sm^r10900)
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In Table 7 are presented results of quantitative transformation experiments with DNAs extracted from transformants in various recipient/donor combinations The theoretical background of experiments of this kind has been discussed elsewhere (*Bovre* 1965a) The upper part of the table shows that following transformation of, let us say, strain A (wild type) by strain B (resistant mutant), the transformant behaves essentially like strain A (resistant *mutant*) as donor in subsequent transformation of strain A (wild type) This is taken to indicate that the lower interstrain than intrastrain transformation fre-

liquefaciens 4663/62 from 1 6 10^{3} to 5 2 10^{-3}, and those with the recipient *Moraxella bovis* 10900 are ranging from 8 0 10^{3} to 1 8 10^{2}

TABLE 6

Transformation of Moraxella nonliquefaciens *and* Moraxella bovis *to Streptomycin Resistance with DNAs from Various Serum Liquefying nonhaemolytic* Moraxella *Strains*

Recipient strain	Donor strain	Recipient count/ml	Interstrain trans-formants/ml	Intrastrain trans formants/ml§	Ratio of inter to intrastrain trans formation
M nonliq 7784	9833	6 0 10^{7}	2 6 10^{2} (26)†	1 8 10^{5} (181)	1 4 10^{3}
	9833	7 5 10^{7}	3 0 10^{2} (30)	2 4 10^{5} (237)	1 3 10^{3}
	L1	6 0 10^{7}	2 2 10^{2} (22)	1 8 10 (181)	1 2 10^{3}
	L1	7 5 10^{7}	3 3 10^{2} (33)	2 4 10^{5} (237)	1 4 10^{3}
	A947(1)	6 0 10^{7}	2 0 10^{2} (20)	1 8 10^{5} (181)	1 1 10^{3}
	A947(1)	7 5 10^{7}	2 4 10^{2} (24)	2 4 10^{5} (237)	1 0 10^{-3}
M nonliq 4663/62	9833	6 0 10^{7}	2 2 10^{2} (22)	6 2 10^{4} (62)	3 5 10^{3}
	L1	6 0 10^{7}	1 0 10^{2} (10)	6 2 10^{4} (62)	1 6 10^{3}
	A947(1)	6 0 10^{7}	1 5 10^{2} (15)	6 2 10^{4} (62)	2 4 10^{3}
	A947(1)	9 4 10^{7}	6 8 10^{2} (68)	1 3 10^{5} (130)	5 2 10^{3}
M bovis 10900	9833	5 8 10^{7}	2 7 10^{2} (27)	1 5 10^{4} (15)	1 8 10^{2}
	9833	1 2 10^{8}	3 6 10^{2} (36)	2 3 10^{4} (23)	1 6 10^{2}
	L1	5 8 10^{7}	1 2 10^{2} (12)	1 5 10^{4} (15)	8 0 10^{3}
	L1	1 2 10^{8}	2 2 10^{2} (22)	2 3 10^{4} (23)	9 6 10^{3}
	A947(1)	5 8 10^{7}	1 5 10^{2} (15)	1 5 10^{4} (15)	1 0 10^{2}
	A947(1)	1 2 10^{8}	2 0 10^{2} (20)	2 3 10^{4} (23)	8 7 10^{3}

Duration of DNA exposure 15 min Selection of transformants at 50 µg streptomycin per ml with parallels at 500 and sometimes also 10 µg per ml § Intrastrain transformants have been scored in simultaneous transformation of the recipient with its own mutant DNA Identical intrastrain transformant counts indicate parallel experiments † Figures in brackets indicate means of 6 8 plate counts (see text)

There seems to be a polarity as regards genetic transfer between *Moraxella bovis* 10900 and the strain A947(1), the compatibility between the two strains being expressed by an 8 times higher ratio when the former strain is receiving the marker (Table 6), as compared with its activity as donor (Table 5) A polarity of that order is the maximum observed by the author in a pair of *Moraxella* or *Neisseria strains* In *Neisseria*, investigations by *Catlin & Cunningham* (1961) frequently revealed even more pronounced polarities of this kind Such polarities are not necessarily of a strictly genetic nature

Means of plate counts listed in Tables 5 and 6 were calculated either from counts at 50 µg streptomycin per ml or collectively for this selection concentration and one or both of the concentrations 10 and 500 µg per ml In the latter instances, no single observation differed by more than 3 times (in most cases less than 2 times) the standard deviation of counts at presumptively uncritical streptomycin concen-

50 60 years, it may be questioned whether a group of serum liquefying moraxellae, significantly more fastidious than the group represented by the strains A947(1) and 11748, does really exist One is inclined to consider the two strains as representatives of *Moraxella lacunata*

In terms of streptomycin resistance transformation, semiquantitative experiments indicate a first degree compatibility between the strains 7911, 9833 and 9985 The same holds for the interrelations between the strains E1 and L1 It is interesting to note that the latter two strains represent distinct serological groups of guinea pig moraxellae (*Ryan* 1964) The strains A947(1) and 11748 are also very closely interrelated in these terms, when examined quantitatively

As far as can be deduced from the semiquantitative and quantitative experiments in Tables 3 and 4, the ratios of inter to intrastrain transformation between the respective sets of highly compatible strains seem to be a little lower than ratios indicating a first degree relationship (*Bovre* 1964 b) Three clusters of strains seem to exist, strikingly in accordance with conventional characteristics and origin However, the 7 serum liquefying, nonhaemolytic strains may all be close enough to each other to be considered as a single *almost* homogeneous entity, as expressed by streptomycin resistance transformation

The relations in these terms between the serum liquefying nonhaemolytic strains and *Moraxella nonliquefaciens* are considered to be essentially similar to those between the former strains and *Moraxella bovis* Also in these experiments, however, more transformable strains would have been preferable

It can now be stated that *Moraxella nonliquefaciens*, *Moraxella bovis* and the serum liquefying nonhaemolytic moraxellae represent three distinct entities in terms of streptomycin resistance transformation The relatively efficient genetic exchange between any pair of these entities may be expressed by ratios of inter to intrastrain transformation of the order 10^{-3} to 10^{-7} (see also *Bovre* 1965a) The group of [illegible] ver- [illegible] the [illegible] present *Moraxella lacunata*, as suggested the relation between the latter and *Moraxella liquefaciens* is very close in these terms

The strains 19116.51 and 752.52 are practically incompatible in terms of streptomycin resistance transformation with the serum liquefying nonhaemolytic moraxellae, as they are with *Moraxella nonliquefaciens* and *Moraxella bovis* The results are particularly valid in the case of *strain 19116.51* which has been used as recipient and donor in combination with each of the three recognized genetic transfer entities of *Moraxella*

quencies noted in Tables 5 and 6 are mainly an expression of DNA heterologies outside the marker loci in donor and recipient bacteria

In the lower part of Table 7 it is indicated that DNA extracted from the mentioned transformant contains structures specific for strain B According to *Schaeffer* (1958), these structures were not detected in the former experiments because their effect was "overshadowed by extensive homologies outside the marker locus When the transformant DNA is allowed to transform strain B (wild type) instead of strain A (wild type), these strain B specific structures express their presence by a 4–7 times *increase in transformation frequency* as compared with that elicited by strain A (resistant mutant) In line with this theory, it is reasonable to suppose that the transformant has been subject to a wider change of DNA constitution than just one of the marker locus In the transformation between these bacteria frequency *differences of the low order 4–7 times are therefore most probably* greater than the ones eventually caused by heterologies of the marker loci alone, in an otherwise controlled procedure

DISCUSSION AND CONCLUSION

In terms of conventional criteria the 5 least fastidious, serum liquefying, nonhaemolytic strains are in accordance with the description of *Moraxella liquefaciens* in Bergey's manual (*Murray* 1957) The findings of *Ryan* (1964) that his strains from healthy guinea pig conjunctiva are indistinguishable from other *Moraxella liquefaciens* strains in common tests, are corroborated

The strains A947(1) and 11748 are able to grow at 25° C, although growth was clearly retarded as compared with the other *Moraxella* strains mentioned *Moraxella lacunata* is generally considered not to grow at "room temperature" (*Scarlett* 1916 a o) *Audureau* (1940) stated that the temperature range for growth of this species is 28–37° C This criterion does not seem sharply distinctive, however (*Oeding*, review 1946) *Henriksen* (1952) found at least some growth at 20° C of all his strains, among which the designations *Moraxella lacunata* and diplobacillus of Morax were represented The strains A947(1) and 11748 are definitely more fastidious than the other strains included in these studies, also when compared with strains considered to be typical *Moraxella nonliquefaciens* The strain 11748 and two other strains with similar fastidiousness had the designation *Moraxella lacunata* No strain of the fastidious group was completely unable to grow in the absence of serum or ascites *Mc Nab* (1904), examining several Morax-Axenfeld organisms, found weak growth in some media without the addition of serum or ascites *Scarlett* (1916) noted that the diplobacillus of Morax grows feebly in ordinary bouillon Other authors have had similar experiences, as reviewed by Oeding (1946) Considering also the evolution of culture media during the passed

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SUMMARY

7 serum-liquefying, nonhaemolytic *Moraxella* strains were included in the studies. In conventional tests, 5 strains were in accordance with *Moraxella liquefaciens* (2 of these were guinea pig isolates), and the remaining 2 strains probably represented *Moraxella lacunata*.

Totally, inter- to intrastrain streptomycin resistance transformation ratios ranged from approximately 1/10 to 1. Within these limits, however, three clusters of strains were represented, each with ratios close to 1. These clusters consisted of the 2 probable *Moraxella lacunata* strains, the 2 guinea pig isolates and the remaining 3 *Moraxella liquefaciens* strains, respectively.

Inter- to intrastrain ratios of streptomycin resistance transformation between the serum-liquefying, nonhaemolytic moraxellae and *Moraxella nonliquefaciens* ranged from 1.0×10^{-3} to 5.2×10^{-3}, whereas the ratios between the former strains and *Moraxella bovis* ranged from 1.2×10^{-3} to 1.8×10^{-2}.

It is stated conclusively that *Moraxella nonliquefaciens*, *Moraxella bovis* and the serum-liquefying, nonhaemolytic moraxellae represent three distinct entities in terms of streptomycin resistance transformation. The relations between any pair of these entities are essentially similar and may be expressed by ratios of inter- to intrastrain transformation of the relatively high order 10^{-3} to 10^{-2}. If the assumed *Moraxella lacunata* strains examined are representative, *Moraxella lacunata* and *Moraxella liquefaciens* seem to be even more closely related to each other than to *Moraxella nonliquefaciens* and *Moraxella bovis*.

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into the plastic Petri dishes 90 mm in diameter The layer of medium was 6 mm thick

Determination of Antibacterial Effect

a) *Dilution* method with solid media, "plate dilution" (*Regn et al* 1963) Each series consisted of 10 12 plates containing two fold serial dilutions of the substance to be examined A loopful (0 01 ml platinum loop) of bacterial suspension in placenta infusion broth with 25 per cent ascitic fluid was spread over 1-2 sq cm of the surface of the plate The suspensions contained about 2 × 10^9 bacteria/ml (*Neis seriae*) or approximately 10^7 bact/ml of bacteria with denser growth than *Neis seriae* The lowest concentration of the test substance in the plates in which growth was distinctly inhibited was called the minimum inhibitory concentration (MIC)

b) *Diffusion method* The plates were inoculated with a bacterial suspension just strong enough to give growth of coalescent colonies After inoculation the plates were dried at 37° C for 30 minutes Filter paper discs, 13 mm in diameter, were placed on the surface of the plate and soaked with 0 03 ml of 0 5 per cent acetazol amide

Temperature and Ambient Gases

Gonococci were incubated in closed plastic containers with a CO_2-concentration of about 10 per cent prepared from sodium bicarbonate and sulphuric acid Other bacteria were incubated in the air All cultures were incubated at 37° C for 20-24 hours

Carbonic anhydrase inhibitor The acetazolamide preparation used was Diamox (Lederle) All the solutions of acetazolamide were prepared in sterile 1/15 M phos phate buffer with a pH of 7 2

Sulphonamide 6 sulphanilamide 2,4 dimethyl pyrimidine, sulphisomidine (Elko sin Ciba) was used as representative of the sulphonamides which exert their bac teriostatic effect by inhibiting PABA The same solvents were used as for acetazol amide

P aminoben oic acid PABA (Merck Darmstadt) The abovementioned phosphate buffer was used as solvent

RESULTS

Effect of Acetazolamide on Growth of Bacteria

Neisseria gonorrhoeae 48 gonococcal strains were studied for their sensitivity to acetazolamide by the plate dilution method (Table 1) The results show that acetazolamide inhibits the growth of gonococci The minimum inhibitory concentration varied between 3 6 and 114 µg ml The range of variation of sensitivity to acetazolamide from one occasion to another was only narrow

TABLE 1

The ᵐ -

Aceta Num	strains	1	0	8	19	19	1	0	0	0

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The Institute of Bacteriology, University of Lund, Sweden

THE EFFECT OF CARBONIC ANHYDRASE INHIBITOR ON THE GROWTH OF *NEISSERIAE*

By

A FORKMAN and A-B LAURELL

Received 14 v 65

The carbonic anhydrase inhibiting property of sulphanilamide and of some other sulpha derivatives was demonstrated in 1940 by *Mann & Keilin* This property proved to be dependent on the intactness of the sulphonamide group Substitution of one or both hydrogen atoms in the sulphonamide group resulted in loss of the inhibitory effect (*Mann & Keilin* 1940, *Lock et al* 1941, *Krebs* 1948) Such substitution, on the other hand, did not abolish the antibacterial activity which is dependent on the intactness of the amino group (*Mann & Keilin* 1940) The bacteriostatic effect and the carbonic anhydrase inhibiting effect of sulphanilamide are thus two separate properties of one and the same molecule (*van Goor* 1943, *Havinger et al* 1946)

Since the discovery of the carbonic anhydrase inhibiting capacity of sulphanilamide, a large number of sulphonamides have been synthetized with higher inhibiting capacity A strong carbonic anhydrase inhibitor is 2-acetyl-amino 1,3,4-thiodiazol-5-sulphonamide (acetazolamide) (*Miller et al* 1950, *Woodford et al* 1961) As in most carbonic anhydrase inhibiting sulphonamides, in acetazolamide the amino group is substituted

This paper is concerned with the effect of acetazolamide on the growth of

1 a) *Neisseria gonorrhoeae*
  b) *Neisseria meningitidis*
  c) *Neisseria flava*
2 Bacteria other than *Neisseria*, and
3 *Neisseria* in the presence of PABA

MATERIALS AND METHODS

Neisseria gonorrhoeae Of the strains studied 38 had been freshly isolated from samples sent for bacteriological analysis The remaining 10 were old laboratory strains

Neisseria meningitidis 5 freshly isolated strains from cases of meningococcal meningitis and two laboratory strains

Neisseria flava 5 strains freshly isolated from throat swabs

The Neisseria strains were differentiated according to conventional diagnostic methods

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rlanson (1951) but
without glucose The final pH of the medium was 7,3–7,4 The medium was poured into the plastic Petri dishes 90 mm in diameter The layer of medium was 6 mm thick

Determination of Antibacterial Effect

a) *Dilution method* with solid media, "plate dilution" (*Reyn et al* 1963) Each series consisted of 10–12 plates containing two fold serial dilutions of the substance to be examined A loopful (0 01 ml platinum loop) of bacterial suspension in placenta infusion broth with 25 per cent ascitic fluid was spread over 1–2 sq cm of the surface of the plate The suspensions contained about 2 × 10⁸ bacteria ml (*Neisseriae*) or approximately 10⁷ bact./ml of bacteria with denser growth than *Neisseriae* The lowest concentration of the test substance in the plates in which growth was distinctly inhibited was called the minimum inhibitory concentration (MIC)

b) Diffusion method The plates were inoculated with a bacterial suspension just strong enough to give growth of coalescent colonies After inoculation the plates were dried at 37° C for 30 minutes Filter paper discs, 13 mm in diameter, were placed on the surface of the plate and soaked with 0 03 ml of 0 5 per cent acetazolamide

Temperature and Ambient Gases

Gonococci were incubated in closed plastic containers with a CO_2-concentration of about 10 per cent prepared from sodium bicarbonate and sulphuric acid Other bacteria were incubated in the air All cultures were incubated at 37° C for 20–24 hours

Carbonic anhydrase inhibitor The acetazolamide preparation used was Diamox (Lederle) All the solutions of acetazolamide were prepared in sterile 1/15 M phosphate buffer with a pH of 7 2

Sulphonamide 6 sulphanilamide 2 4 dimethyl pyrimidine, sulphisomidine (Elkosin Ciba) was used as representative of the sulphonamides which exert their bacteriostatic effect by inhibiting PABA The same solvents were used as for acetazolamide

P aminobenzoic acid PABA (Merck Darmstadt) The abovementioned phosphate buffer was used as solvent

RESULTS

Effect of Acetazolamide on Growth of Bacteria

Neisseria gonorrhoeae 48 gonococcal strains were studied for their sensitivity to acetazolamide by the plate dilution method (Table 1) The results show that acetazolamide inhibits the growth of gonococci The minimum inhibitory concentration varied between 3 6 and 114 µg/ml The range of variation of sensitivity to acetazolamide from one occasion to another was only narrow

TABLE 1

The Minimum Inhibitory Concentrations of Acetazolamide Determined for 48 Strains of Neisseria gonorrhoeae

Acetazolamide µg/ml	3 6	7 2	14,3	28 5	57	114	228	456	912
Number of strains	1	0	8	19	19	1	0	0	0

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The Institute of Bacteriology, University of Lund, Sweden

THE EFFECT OF CARBONIC ANHYDRASE INHIBITOR ON THE GROWTH OF *NEISSERIAE*

By

A. FORKMAN and A. B. LAURELL

Received 14.x.65

The carbonic anhydrase inhibiting property of sulphanilamide and of some other sulpha derivatives was demonstrated in 1940 by *Mann & Keilin*. This property proved to be dependent on the intactness of the sulphonamide group. Substitution of one or both hydrogen atoms in the sulphonamide group resulted in loss of the inhibitory effect (*Mann & Keilin* 1940, *Roel et al.* 1941, *Krebs* 1948). Such substitution on the other hand did not abolish the antibacterial activity which is dependent on the intactness of the amino group (*Mann & Keilin* 1940). The bacteriostatic effect and the carbonic anhydrase inhibiting effect of sulphanilamide are thus two separate properties of one and the same molecule (*van Goor* 1943, *Havinger et al.* 1946).

Since the discovery of the carbonic anhydrase inhibiting capacity of sulphanilamide a large number of sulphonamides have been synthetized with higher inhibiting capacity. A strong carbonic anhydrase inhibitor is 2-acetylamino-1,3,4-thiodiazol-5-sulphonamide (acetazolamide) (*Miller et al.* 1950, *Woodford et al.* 1961). As in most carbonic anhydrase inhibiting sulphonamides, in acetazolamide the amino group is substituted.

This paper is concerned with the effect of acetazolamide on the growth of

1. a) *Neisseria gonorrhoeae*
   b) *Neisseria meningitidis*
   c) *Neisseria flava*
2. Bacteria other than *Neisseria*, and
3. *Neisseria* in the presence of PABA.

MATERIALS AND METHODS

Neisseria gonorrhoeae. Of the strains studied, 38 had been freshly isolated from samples sent for bacteriological analysis. The remaining 10 were old laboratory strains.

Neisseria meningitidis. 5 freshly isolated strains from cases of meningococcal meningitis and two laboratory strains.

Neisseria flava. 5 strains freshly isolated from throat swabs.

The Neisseria strains were differentiated according to conventional diagnostic methods.

influenzae and 11 strains of *E coli* were studied by the plate dilution method Most of these strains were resistant to 912 μg acetazolamide/ml None of the strains were inhibited before the concentration of acetazolamide reached 228 μg ml (Table 3)

TABLE 4

The Minimum Inhibitory Concentrations of Acetazolamide and Sulphisomidine Determined for 35 Different Bacteria. The Gonococci Were Cultured in Air Containing ca. 10 per Cent CO_2 *the other Bacteria Were Cultured in Air without Added* CO_2

	Strain	Acetazolamide (μg/ml)	Sulphisomidine (μg/ml)
Neisseria gonorrhoeae	1	57	7.2
"	2	57	< 3 6
"	3	57	7 2
	4	57	3 6
"	5	57	< 3 6
"	6	28 5	7 2
"	7	28 5	7.2
Neisseria meningitidis	1	0 9	0 9
"	2	0 9	0 9
	3	0 9	0 9
"	4	0 45	0 9
"	5	0 45	0 45
"	6	0 45	0 9
	7	0 45	0 45
Neisseria flava	1	0 45	3 6
	2	0 45	3 6
"	3	0 45	3 6
"	4	0 45	3 6
"	5	0 45	> 14 3
Haemophilus influenzae	1	912	7 2
	2	456	7 2
	3	456	14 3
	4	456	7 2
Staphylococcus aureus	1	> 1824	114
"	2	> 1824	7 2
	3	> 1824	14 3
	4	> 1824	114
	5	> 1824	114
	6	> 1824	14 3
"	7	> 1824	57
Escherichia coli	1	> 1824	114
	2	> 1824	114
"	3	> 1824	114
	4	1824	57
"	5	228	28 5

Effect of Acetazolamide on Neisseria in Presence of PABA

Though acetazolamide has a substituted amino group and therefore lacks the antibacterial properties of other sulphonamides which is due to competitive PABA — inhibition (*Woods* 1940) it was decided to

Twenty five of the strains were also examined with the diffusion method, and all showed a zone of inhibition of growth around the discs The zones around 7 strains were only diffusely outlined so that the diameter could not be measured with any accuracy The diameter of the zones in the remaining 18 tests ranged between 21 and 38 mm

Thus also the diffusion method demonstrated the inhibitory effect of acetazolamide on the growth of gonococci But when tested by this method the sensitivity to the drug varied substantially both from one occasion to another and from strain to strain

Neisseria meningitidis Seven strains were studied by the plate dilution method and the diffusion method (Table 2) The experiment showed that acetazolamide has a strong inhibitory effect on the growth of meningococci When tested by the plate dilution method the strains appeared roughly equally sensitive (MIC 0 45 0 9 µg ml) When studied by the *diffusion method, however*, the sensitivity appeared less uniform the zone of inhibition varying somewhat from strain to strain (36 55 mm)

TABLE 2

The Minimum Inhibitory Concentrations of Acetazolamide Determined for 7 Strains of Neisseria meningitidis and 5 Strains of Neisseria flava

Acetazolamide µg/ml	< 0 11	0 11	0 22	0 45	0 9	> 0 9
N meningitidis	0	0	0	3	4	0
N flava	0	0	0	5	0	0

Neisseria flava The strains were studied by the plate dilution method and the diffusion method These apathogenic *Neisseria* strains from the throat proved as sensitive to acetazolamide (MIC 0 45 µg ml) as did *meningococci (Table 2) Also in this experiment* the plate dilution method gave more constant values than the diffusion *method* which showed inhibition zones varying between 43 and 53 mm in diameter

TABLE 3

The Minimum Inhibitory Concentrations of Acetazolamide Determined for some Bacteria not Belonging to Neisseria

	Number of strains inhibited by			
	< 456 µg/ml	456 µg ml	912 µg/ml	> 912 µg ml
Diplococcus pneumoniae	0	1	0	3
Staphylococcus aureus	0	2	0	10
Haemophilus influenzae	0	3	2	3
Escherichia coli	1 (228 µg/ml)	1	0	9

Other bacteria than Neisseriae Four strains of *Diplococcus pneumoniae*, 12 strains of *Staphylococcus aureus* 8 strains of *Haemophilus*

Since acetazolamide has a substituted amino group one would hardly expect it to possess any antibacterial activity. On comparison between acetazolamide and a sulphonamide with ordinary bacteriostatic effect through the competition with PABA, the sensitivity of the bacteria to acetazolamide was found to vary independently of their sensitivity to sulpha. Moreover, the sensitivity of the *Neisseria* strains to acetazolamide was not affected by addition of PABA to the medium. The results of the experiments suggest that the mechanism of the inhibitory effect of acetazolamide on the growth of the *Neisseria* strains studied differs from that of the sulphonamides.

Acetazolamide is an affective carbonic anhydrase inhibitor. It is possible that it is this property that acetazolamide inhibitis the growt of the *Neisseria* strains studied. Preliminary experiments showed that a crude preparation of carbonic anhydrase is a growth promoting factor for gonococci and that this growth stimulating effect can be abolished by equilibrating carbonis anhydrase and acetazolamide added in the medium.

Investigations in progress have shown that the inhibitory effect of acetazolamide varies with the ambient CO_2-concentration. *N. meningitidis* and *N. flava* have proved sensitive to acetazolamide only at low concentration of CO_2 in the ambient gases. In excess of CO_2 the growth of these bacteria is not substantially affected by the presence of carbonic anhydrase inhibitor. When cultured in an ambient atmosphere containing 10 per cent CO_2 the growth of *N. gonorrhoeae* is inhibited by acetazolamide. This may suggest that *N. gonorrhoeae* is more dependent on carbonic anhydrase than the other types of *Neisseria* studied. It is possible that the variation of the zones of inhibition in the plates studied by the diffusion method can be explained by variation in the CO_2-concentration of the ambient gases.

Another point deserving attention is the amount of carbonic anhydrase in *Neisseria* compared with that in types of bacteria highly resistant to carbonic anhydrase inhibiting substances. A survey of the literature revealed only a few reports on carbonic anhydrase in bacteria. *Main & Locke* (1944) reported that type I pneumococcus and *Staphylococcus aureus* did not produce carbonic anhydrase. [illegible] ... the other bacteria investigated (*streptococcus salivarius*, *Lactobacillus acidophilus* and some other non pathogenic *Neisseria* strains) showed no or little activity.

Investigations are under way to determine the carbonic anhydrase content of the strains of *Neisseria* and other bacteria used in this study. Studies are also in progress to elucidate the mechanism of the antibacterial effect of carbonic anhydrase inhibitor on *N. gonorrhoeae*, *N. meningitidis* and *N. flava*.

compare the effect of acetazolamide with that of a noncarbonic anhydrase inhibiting sulphonamide (sulphisomidine).

Determinations of the lowest inhibiting concentrations of acetazolamide and sulphisomidin (Elkosin) were made by the plate dilution method Seven *gonococcal strains, 7 meningococcal strains, 5 N flava* strains, 4 *Haemophilus influenzae* strains, 7 *Staphylococcus aureus* strains and 5 *E coli* strains were used (Table 4) The sensitivity of the bacteria to acetazolamide proved to vary irrespectively of their sensitivity to sulphonamide

Acetazolamide and sulphisomidine were also studied by the plate dilution technique in medium containing PABA in various concentrations Four gonococcal strains were used (Table 5) The addition of PABA markedly suppressed the antibacterial effect of sulphisomidine but had no significant influence on the antibacterial effect of acetazolamide

TABLE 5

The Minimum Inhibitory Concentrations of Acetazolamide and Sulphisomidine with and without PABA in the Culture Medium for 4 Strains of Gonococci

Strain	MIC sulphisomidine (µg/ml)		MIC acteazolamide (µg/ml)	
	PABA 0 µg/ml	PABA 10 µg/ml	PABA 0 µg/ml	PABA 10 µg/ml
1	1 8	456	28 5	57
2	3 6	456	28 5	57
223	7 2	456	28 5	28 5
235	3 6	228	28 5	28 5

The result of the experiments argues against the antibacterial effect of acetazolamide being essentially the same as that of a non carbonic anhydrase inhibiting sulphonamide

DISCUSSION

Investigation of 48 gonococcal strains showed that acetazolamide can inhibit the growth of these bacteria Corresponding studies of meningococci and *Neisseria flava* showed that acetazolamide has a strong antibacterial effect also on these two species of *Neisseria* The lowest inhibiting concentration, as assessed by the plate dilution method varied only slightly from strain to strain of the same species and from one occasion to another In experiments using the diffusion method with discs containing *acetazolamide*, on the other hand, the sizes of the zones of inhibition varied substantially not only from strain to strain of the but also from one occasion to another No explanation

All the other bacteria studied were ...

Acta path et microbiol scandinav 65 457–466 1965

International Escherichia Centre, Statens Seruminstitut, Copenhagen Denmark

IN VITRO STUDIES ON THE FATE OF ANTIGEN

5 *The Uptake of I^{131} HSA by Mouse Exudate Cells in the Presence of Normal Serum and Anti-HSA Serum*

By

J M RHODES

Received 22 vi 65

Considerable information has accumulated on the fate of soluble antigens in normal and immune animals employing radioactive labelled antigens (see e g review by *Campbell & Garvey* (3))

As *Humphrey* (10) has pointed out, two processes for the ingestion of antigen may occur concurrently, a non-selective ingestion of soluble fluid and a selective removal of particulate material by the reticulo-endothelial system notably the Kupffer cells of the liver

Various authors (13, 5, 12) have demonstrated that rabbit antigen/antibody complexes formed at equivalence are taken up and broken down by guinea pig exudate cells *in vitro* The other main sources of cells employed for this type of *in vitro* study has been from rabbits and mice Each species of animal was usually first injected intraperitoneally with an inflammatory agent in order to increase the yield of macrophages *Cohn & Benson* (7) have demonstrated that macrophages appearing in the peritoneal cavity of mice after intraperitoneal injection of an irritant contain larger quantities of enzymes than macrophages from normal mice In view of this fact it seemed of interest to investigate the degree of phagocytosis of antigen-antibody complexes in exudate cells harvested at various time intervals after the injection of an irritant

Soluble antigen-antibody complexes are taken up *in vitro* by spleen cells (11) by exudate cells (12) and antigen alone by exudate cells using either radioactive labelled antigen (1) or fluorescein labelled antigen (9) "Native" proteins were shown by phase contrast microscopy to be taken up by exudate cells from rabbits and guinea pigs (4) and by human leucocytes (17) Since exudate cells are capable of ingesting soluble antigen and phagocytosing particulate antigen, the exudate cells employed in this series of experiments were also investigated for their capacity to engulf soluble matter, simultaneously with their ability to phagocytose particulate matter

The author wishes to acknowledge the excellent technical assistance of Bente Kjær Jensen

SUMMARY

1 A carbonic anhydrase inhibiting sulphonamide acetazolamide proved to inhibit the growth of various types of *Neisseria*, but not of other bacteria studied

2 The growth inhibition is not the result of competitive inhibition of PABA The mechanism of the inhibition is unknown but might be due to inhibition of carbonic anhydrase of the bacteria

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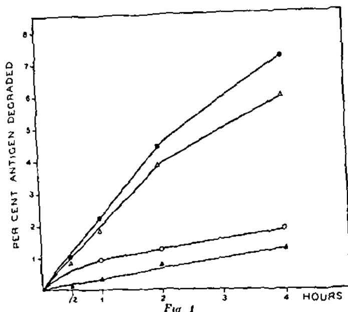


Fig 1

The breakdown of I^{131} HSA in the presence of precipitating rabbit anti HSA serum and mouse peritoneal exudate cells harvested at various time intervals after intra peritoneal injection of proteose/peptone

▲——▲ control exudate cells no injection
○——○ 1 day exudate cells
●——● 2 day exudate cells
△——△ 3 day exudate cells

I^{131} HSA antibody complex, whereas 1 day exudate cells and control exudate cells (from non injected mice) are not as effective There is not a great deal of difference in the behaviour of the 2 and 3 day exudate cells or the 1 day and control exudate cells, see Fig 1, Table 1 It is impossible to measure the percentage uptake of the antigen-antibody complex since the radioactive complex is centrifuged down with the cells These results are the average of duplicate experiments

Uptake and Breakdown of I^{131} HSA by Mouse Cells

I^{131} HSA alone (i e incubated with cells in the presence of normal rabbit serum) is broken down slightly by the various types of exudate cells and apparently somewhat more rapidly by 3 day exudate cells see Table 1, Fig 2 These results are taken from a typical experiment, but other results from similar experiments suggest that there is not a great deal of difference in the breakdown of antigen by the various types of exudate cells

There is considerable variability in the percentage uptake of I^{131} HSA in the presence of normal rabbit serum, from 0 005 0 1 per cent of the total I^{131} radioactivity added to the cells can be measured on them after 4 hours

MATERIAL AND METHODS

Antigen

Human serum albumin I^{131} (I^{131} HSA) specific activity usually 0.023 mC/mg was obtained repeatedly from the Radiochemical Centre Amersham Buckinghamshire England

Antiserum

Pool 702 prepared by injecting rabbits 3 times weekly for 3 weeks with human serum albumin (HSA) 10 mg/ml intravenously The animals were bled 8 days after the last injection Only sera with high titres of precipitating antibodies were used for the pool

Animals

Inbred C3H mice bred at the Statens Seruminstitut The mice were 4 6 months old of both sexes

Preparation of Cells

Mice were injected with 2 ml proteose/peptone 10 per cent in saline intraperitoneally Cells were harvested 1 2 and 3 days after injection by washing out the peritoneal cavity with 3 ml saline containing 1 20 000 heparin and 5 per cent normal rabbit serum The cells were washed twice in Gey's medium containing 10 per cent normal rabbit serum (NRS) The cells were suspended in Gey s solution and adjusted to contain 2×10^7 cells/ml

Differential Cell Counts

All cell suspensions were adjusted to 10^6 cells/ml 0 1 ml was spun down by centrifugal force onto a microscope slide (2) The cells were then fixed in alcohol and stained with haemotoxylin eosin Giemsa or Wright s stain

For the purpose of this article large macrophages and the lymphocyte macrophage intermediate are listed under the heading of macrophages since both types of cells phagocytosed carbon particles

Experimental Procedure for the Uptake and Breakdown of I^{131} HSA by Mouse Exudate Cells

To 0 1 ml (2×10^6 cells) of the cell suspensions prepared as above was added 0 4 ml Gey s solution and 0 1 ml NRS or 0 1 ml pool 702 + 20 µl I^{131} HSA Appropriate controls without cells were also prepared The mixtures were incubated at 37° C for various time intervals up to 4 hours shaking well at half hourly intervals At the allotted time the cells were centrifuged down at 300 rpm for 3 minutes and washed 4 times with Gey s solution Finally the cells were placed on a small filter paper on an aluminium planchette for radioactive assay The percentage of original radioactivity taken up by the cells is calculated from the figure obtained

Evaluation of the breakdown of I^{131} HSA is based on the measurement of non protein bound radioactivity in the medium at the various time intervals i e material soluble in 5 per cent trichloracetic acid An aliquot of the cell supernatant after addition of TCA was also placed on aluminium planchettes for radioactive assay

For further details of this procedure see *Sorkin & Boyden* (14)

Isotope Assay

The radioactivity of the samples dried at 50 60° C was measured in a Geiger Muller tube coupled to an electronic counter (supplied by Philips PW 4032) The background was 26 cp m

RESULTS

Uptake and Breakdown of I^{131} HSA Antibody Complex by Mouse Cells

Mouse exudate cells collected 2 days and 3 days after the intraperitoneal injection of proteose/peptone rapidly break down a precipitated

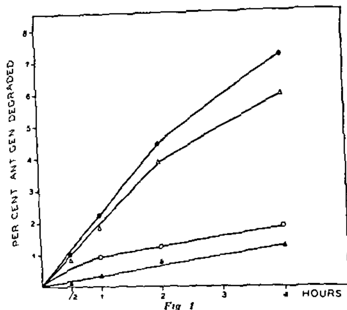


Fig 1

abbit anti HSA serum
intervals after intra

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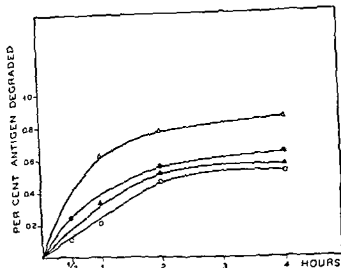
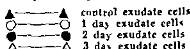


Fig 2

The breakdown of I131 HSA in the presence of normal rabbit serum and mouse peritoneal exudate cells harvested at various time intervals after intraperitoneal injection of proteose/peptone



In the experiment shown in Table 1 only 0 026 per cent was found on the 3 day exudate cells after 4 hours, whereas none could be measured on the other exudate cells This may be partially due to experimental error in isotope assay when such low amounts of radioactive antigen are offered to the cells, the state of the cells themselves, or the state of the radioactive antigen which may alter for each new batch used The use of an antigen of higher specific activity might alter the picture considerably

Differential Cell Counts

Table 2 illustrates the differences in the cell composition of the 4 types of cell exudates

The percentage of macrophages is similar in the control cells and in the 1 day exudate cells, and in the 2 day and 3 day exudate cells, respectively However, 1 day after injection of proteose/ peptone the lymphocytes disappear to be replaced by polymorphonucleocytes, which also recede after 2 days This is similar for the pattern already described for guinea pig exudate cells after the intraperitoneal injection of an inflammatory agent (15)

The various types of cells found in these exudates are illustrated in Figs 3 a d after staining with haematoxylin eosin

TABLE 1

Uptake and Breakdown of I131 HSA in the Presence of Mouse Exudate Cells

Days after injection of proteose/peptone	Time of incubation 37° C	Serum added (0 1 ml)	Cell deposit washed 4 times % uptake	Breakdown of antigen %	Serum added (0 1 ml)	Cell deposit washed 4 times % uptake	Breakdown of antigen %
0	½	Normal rabbit	–	0 12	Rabbit anti HSA	ppt cannot be measured	0 05
	1			0 34			0 42
	2			0 52			0 78
	4			0 55			1 25
1	½	Normal rabbit			Rabbit anti HSA	ppt cannot be measured	0 21
	1			0 21			0 9
	2			0 47			1 25
	4			0 52			0 9
2	½	Normal rabbit	–	0 26	Rabbit anti HSA	ppt cannot be measured	0 95
	1			0 19			2 2
	2			0 54			4 4
	4			0 63			7 2
3	½	Normal rabbit	0 003	0 25	Rabbit anti HSA	ppt cannot be measured	0 8
	1		0 015	0 63			1 9
	2		0 023	0 78			3 9
	4		0 026	0 86			5 9

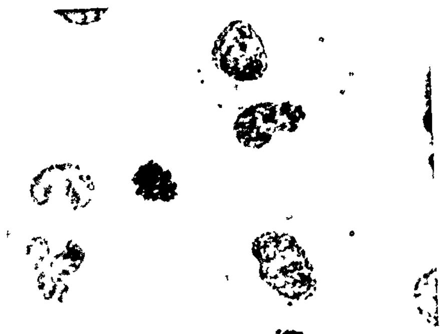


Fig. 3c

Mouse peritoneal cells collected 2 days after injection of [illegible]/peptone consisting mainly of macrophage cells

TABLE 2

Cell Count of Mouse Macrophages

	Macrophage cells	Lymphocytes	Polymorphonucleocytes	Mast cells	Eosinophils
	%	%	%	%	%
Control	42 59	54 35		5–7	
1 day exudate	38 49	3 6	56–46	1 4	4 6
2 day exudate	76–80	4 6	3 6	3 4	4 6
3 day exudate	84 85	4 6	0–4	2 3	3 4

DISCUSSION

It is a well known fact that both polymorphonucleocytes (PMN) and macrophages actively phagocytose particulate matter such as bacteria, erythrocytes. In addition macrophages also break down I^{131} HSA antibody complexes to acid soluble products (13).

The experiments described here *indicate that mouse exudate cells* harvested 3 days following intraperitoneal injection of an irritant and consisting mainly of macrophages break down antigen-antibody com

Fig 3a

Normal mouse peritoneal cells, consisting of mast cells large granular cells to left lymphocytes and mononuclear cells

Fig 3b

Mouse peritoneal cells, collected one day after injection of proteose/ peptone consisting of polymorphonucleocytes and macrophage cells

PMN but is retained in the macrophage It should be borne in mind that this discrimination by the two types of cells may not be applicable to antigen/antibody complexes, but this point is open to experimentation Since PMN cells do not appear to destroy the HSA-complexes, it seems more conceivable to assume that the 1 day exudate cells contain about the same amount of hydrolases as the control cells

Holtzer & Holtzer (9) have stated that all types of cells in rabbit, guinea-pig, and rat exudates pinocytose fluorescent, soluble, antigens *Chapman Andresen* (4) also demonstrated that 1 day exudate cells from rabbits, and guinea-pigs consisting mainly of PMN, pinocytosed various soluble proteins Our observations are in agreement with these results —see Fig 2 which illustrates the breakdown of I^{131} HSA in the presence of normal rabbit serum Control, 1 day, and 2 day exudate cells show no difference in the per cent uptake of I^{131} HSA, whereas the 3 day exudate cells give a slightly higher value However, from repeated experiments, the general impression obtained is that there is no significant difference in the degree of ingestion of I^{131} HSA by 1, 2 and 3 day exudate cells

Under these experimental conditions, a plateau of uptake of antigen was reached after incubation for 4 hours at 37° C This agrees with the pattern observed *in vivo* after the intraperitoneal injection of small amounts of fluorescent HSA (own observations) Increase in the concentration of HSA can, of course, saturate the cells *in vivo* after a much shorter period

These experiments, in agreement with those of other workers (1, 4, 6, 7, 8, 9, 11, 12, 16) indicate that macrophages not only phagocytose particulate matter but also ingest a soluble protein It has been suggested (see *Gropp* (8)) that phagocytosis and pinocytosis (ingestion of soluble matter) may be the same process seen at different microscopic levels Both processes take place rapidly in macrophages (8) As shown in the present experiments, in the presence of rabbit anti-HSA serum normal mouse cells break down 50 per cent more I^{131} HSA than in the presence of NRS This may merely mean that more antigen is taken up by phagocytosis via a particulate complex, than is taken up by pinocytosis Alternatively, the requisite enzymes for breaking down the antigen may be activated more quickly during phagocytosis

SUMMARY

I^{131} HSA-antibody complexes formed at equivalence are phagocytosed to different degrees by mouse peritoneal cells harvested at different days after the intraperitoneal injection of proteose/peptone

In contrast there was no discernible difference in the rate of ingestion of I^{131} HSA *alone* by the same batches of peritoneal cells



Fig 31
Mouse peritoneal cells collected 3 days after injection of proteose/peptone consisting mainly of macrophage cells

plexes to a greater extent *in vitro* than exudate cells harvested earlier *Cohn & Benson* (7) reported that mouse macrophages collected 4 days after the intraperitoneal injection of a lipopolysaccharide contain larger quantities of hydrolases than macrophages from control mice The result mentioned above may therefore be due to the presence of more enzyme in these cells since an overall increase of 40 50 per cent macrophages in the 3 day exudate cells would not account for the increased percentage breakdown of antigen by these cells

Cells from 1 day exudates are apparently only slightly more efficient than control cells in breaking down the antigen One could argue that the macrophages present in these exudate cells contain only about the same amount of hydrolases as in the control cells which infers that the PMN cells are quiescent However it is established that PMN cells rapidly phagocytose particulate matter at least in the form of bacteria (see e g *Vaughan* (16)) If PMN cells break down such complexes as rapidly or even more rapidly than macrophages one would expect to detect a higher percentage of acid soluble products after incubation with the 1 day exudate cells using the method described here This would be in accordance with the results of *Cohn et al* (6) who demonstrated that *E coli* is treated differently by PMN cells and macrophage cells The immunogenicity of the bacteria is completely destroyed in the

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INCOMPLETE ANTI-D ANTIBODY WITH CHANGED Gm SPECIFICITY

By

JACOB B NATVIG

Received 14 v 65

Red cells sensitized by certain incomplete anti-Rh antibodies are agglutinated by anti Gm or anti-Inv factors in selected human sera Inhibition of agglutination by a serum indicates the presence of a given genetic immunoglobulin character (-factor) At least 9 Gm and 3 Inv factors, controlled by codominant allelic genes at the Gm and Inv loci are known (For references see 19, 24 and 30)

The genetic characters have been useful markers of isolated immunoglobulins in the study of antibody synthesis Isolated myeloma proteins expressed not more than one of the factors Gm (a), Gm (b) or Gm (f), although the donors were positive for two or three factors (11, 17, 18)

Such selective occurrence of Gm factors is also well known in anti-Rh antibodies *Harboe* (8, 9) found that even potent incomplete anti-Rh antibodies from Gm (a—b+) donors often lacked the Gm (b) character *Mårtensson* (18) showed that these antibodies were most often Gm (f+) Many anti Rh antibodies are, however, polyspecific, containing two or more Gm factors

Allen Kunkel & Kabat (2) studied various isolated human γG antibodies from the same individual The majority of the antibodies contained all the genetic factors present in the whole γG globulin of the donor However, *in certain* antibodies the distribution approached the selective occurrence of genetic factors in myeloma proteins

This paper presents observations on

1 An incomplete anti D antibody in which the proportion of Gm antigenic determinants had *changed* in spite of maintained antibody specificity

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8 and 16 agglutinating units of the respective anti Gm sera(slide technique) Proper controls were always included Before typing anti Rh antibodies or anti Gm factors were removed by absorption and treatment by mercaptoethanol respectively

Gc Hp Rh and ABO groupings These tests were performed by Drs *J Lundevall* and *T Reinskou*

Inv typing This test was performed by Dr *T Gedde Dahl*

Titration of anti Rh antibodies To 0 2 ml of serum dilution was added 0 05 ml of 10 per cent O Rh positive red cells After incubation at 37° C for 2 hours the tubes were centrifuged (1000 × g for 30 sec) and a small sample of the red cells was removed for microscopic reading The remaining red cells were washed 4 times and 0 05 ml of antiglobulin serum (K980) diluted 1 32 was added to each tube After 10 minutes at room temperature the tubes were centrifuged and samples taken for microscopic reading

Titration of anti A and anti B antibodies The tests were performed by the tube technique using 1 per cent A Rh negative and 1 per cent B Rh negative red cells respectively

The Waaler Rose test for rheumatoid factor The test was performed according to Tonder (29)

Absorption experiments Sensitized red cells were prepared as described above and tested by antiglobulin serum and various anti Gm sera The same batch of sensitized red cells was divided and used for 3 subsequent absorptions of anti Gm(b) and anti Gm(f) Equal volumes of packed sensitized red cells (1000 × g for 5 min) and anti Gm serum diluted 1 2 were mixed and kept for 2 hours at room temperature Serum exposed to unsensitized red cells served as control A small aliquot of serum was removed after each absorption for testing of activity

Elution of anti D antibodies Red cell stromata were prepared (29 p 111) sensitized with anti D in excess and washed 6 times in large volumes of saline The anti D was eluted by diethylether (25) and the eluate tested in the indirect anti globulin test

Reduction by mercaptoethanol Equal volumes of serum and 0 3 M 2 Mercapto

xed kept over night at room tem

IO group and the Rh Gm and Inv

S V is a healthy woman who has never received a blood transfusion gammaglobulin or any serum and has not suffered from any chronic disease Pregnancy events 1954–1955 One abortion and one extrauterine pregnancy *Aug* 1956 partus I a healthy boy No anti Rh antibodies were detected *Dec* 1957 partus II a girl with erythroblastosis foetalis (direct antiglobulin test positive) Tests for anti Rh antibodies were not performed du

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TABLE 1

Blood Groups and Serum Types of S V and her Family

	Born	ABO	Rh	Gm	Inv
Mother S V	1933	A_2	rr	Gm (a—x—b+f+)	Inv (a—)
Father	1930	A_1	R_1R_2	Gm (a+x—b+f+)	Inv (a—)
Boy	1956	A_2	R_1r	Gm (a+x—b+f+)	Inv (a—)
Girl	1957	O	R r	Gm (a—x—b+f+)	Inv (a—)
Girl	1960	A_1	R_2r	Gm (a x—b+f+)	Inv (a—)

2 The absorption of anti-Gm factors by anti-D sensitized red cells which were not agglutinated by the respective anti-Gm serum
3 The development of an isospecific anti-Gm factor

MATERIALS AND METHODS

Red cells Red cells were obtained from whole blood stored in acid citrate dextrose solution at 4° C for maximum 5 days A single donor provided the O R_1R_2 (CDe cDE) red cells In addition O R'r (Cde cde), and O, R"r (cdE cde) red cells were used for detection of anti C and anti E respectively Before use the cells were washed 4 times and packed at 1000 × g for 2 min (Adams sero fuge)

Incomplete anti D sera Samples of serum S V from two different bleedings in 1957 were kindly provided by Drs *M Harboe O Hartmann H Ørjasæter* and *T Gedde Dahl* Oslo Anti D S V 1957 was used for detection of the original anti Gm(b) J K (8) New serum samples from S V were provided for research purpose in 1964 and 1965 The 1964 and 1965 samples showed similar reaction and for convenience are usually both referred to under the latter Drs *K Halvorsen* Bergen *P Østgård* Stavanger and laboratories abroad kindly provided the other anti Rh sera used The titres of the sera in the indirect antiglobulin test were of minimum 128 or at least 128 times the titres (4 or less) in saline

Anti Gm sera Dr *T Quamme* Hatlestad Hospital Bergen and various laboratories in this country and abroad provided anti Gm sera originating from rheumatoid arthritis patients (Raggs) and from normal individuals (Snaggs) A new sample of the original anti Gm(b) J K (8) was provided through the courtesy of Dr *M Harboe*

Normal sera Sera from healthy blood donors of different Gm types served as controls

Anti human gammaglobulin serum (Coombs serum) Antisera were obtained after immunization of albino rabbits with human serum in complete *Freund's* adjuvant given intramuscularly After absorption with A_1B and O red cells the titre of serum K980 against strongly sensitized red cells was 512 1024

All the sera were stored in small aliquots at −25° C until used

Gammaglobulin Human gammaglobulin 12 per cent solution batch *80779* was kindly provided from AB Kabi Stockholm

Diluent Isotonic phosphate buffered saline pH 7 2 was used All titrations were made twofold and whenever possible master dilutions were prepared

Sensitization of red cells One to four volumes of anti D serum were diluted 1 8 and mixed with one volume of packed red cells After incubation at 37° C for 2 hours the sensitized red cells were washed 4 times in large volumes of saline and used in 1 per cent suspension for tube technique and 0 3 per cent suspension for slide technique The sensitized red cells always gave reproducible titres with the antiglobulin serum and were used the day they were prepared

Agglutination tests Tube technique One drop each of sensitized red cells anti serum and saline were mixed and left at room temperature for 1 hour (2 hours for the anti antibody sera) After centrifugation at 1000 × g for 30 sec each tube was gently agitated and the agglutination was graded as +++, ++ and + at the moment when all red cells were dislodged from the bottom

Slide technique The slide technique was performed according to *Brandtzæg & Mohr's micromodification of Harboe's technique* (3 10) Five microlitres each of sensitized red cells antiserum and saline were mixed on properly washed glass slides 26 × [illegible] cm and incubated at room temperature in a humid chamber under gentle agitation for ½ hour Microscopic readings under low magnification were [illegible] in (27 p 34) (Designations [illegible]

[illegible] bserver not knowing which serum was being tested Similar results were obtained with the two agglutination tests In inhibition experiments the saline was substituted by an equal volume of the material to be tested

The titre of the serum was expressed as the reciprocal of the highest dilution at which at least + agglutination was observed One agglutinating unit was defined as the minimum amount of serum causing agglutination in a standard test

Gm typing The serum was diluted 1 8 and its inhibiting capacity tested against

8 and 16 agglutinating units of the respective anti Gm sera (*slide technique*) Proper controls were always included Before typing anti Rh antibodies or anti Gm factors were removed by absorption and treatment *by mercaptoethanol respectively*

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Girl	1960	A_1	R_0r	Gm (a—x—b+f+)	Inv (a—)

EXPERIMENTS AND RESULTS

Four different samples of serum S V were available In order to exclude possible errors of labelling or mixing of specimens, serum from each of the samples was tested for Gm, Gc, and Hp type and for anti-A and anti-B isoagglutinins All of them were Gm (a—x—b +f+), Gc (2—2), Hp (2—2) and contained anti-B in a titre of 128 to 256 but not anti-A

The four samples of serum S V were tested for the presence of anti Rh antibodies (Table 2). No saline agglutinins against O, R_1R_2 red cells were detected No incomplete anti-C or anti-E antibodies were present (indirect antiglobulin test with O,R'r and O,R"r red cells respectively) The only anti-Rh antibody demonstrated in serum S V against O,R_1R_2 red cells by indirect antiglobulin technique was incomplete anti D The titre of this antibody showed a slight decrease from 1957 to 1965 (Titration of the sera in 20 per cent albumin medium (27, p 187–189) gave titres parallel to those of the indirect antiglobulin test)

TABLE 2

Titre of Anti Rh Antibodies in the Four Samples of Serum S V

Sample of serum S V	Test with O, R_1 R_2 red cells in saline	Test with O, R_1 R_2 red cells in indirect antiglobulin test
S V 12/12-57	< 1	256
S V 28/12-57	< 1	512
S V 11/8 -64	< 1	128
S V 18/1 65	< 1	256

Controls Unsensitized red cells and antiglobulin serum no agglutination O R r or O R"r red cells and each of the four samples of serum S V gave no agglutination in saline or in the indirect antiglobulin test

Agglutination Experiments

Red cells sensitized with the different samples of serum S V were agglutinated equally strong by antiglobulin serum, by anti-Gm (f) and by an anti-antibody (16) (Table 3) Anti-Gm (b) 2535 agglutinated strongly red cells sensitized by S V 1957 while no agglutination occurred when serum S V 1965 was used for sensitization

Further tests included the original anti Gm (b) J K, anti Gm (b) sera from *C Ropartz* (Allatre), *L Mårtensson* (I G) *E van Loghem Langerеis* (2247, 2277, 2357) and 3 other anti-Gm (b) sera Some of these sera were specific for Gm (bα), (bβ) and (bγ) respectively None of these anti Gm (b) sera agglutinated red cells sensitized with serum S V 1965

Experiments were performed with further excess of anti-D antibodies Thirty volumes of serum S V 1965 or 10 volumes of this serum concentrated by polyethylenglycol (14) to an incomplete anti-D titre of 512 were used to sensitize one volume of packed red cells Neither these

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S V 28/12 57	< 1	512
S V 11/8 64	< 1	128
S V 18/1 65	< 1	256

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Experiments were performed with further excess of anti-D antibodies Thirty volumes of serum S V 1965 or 10 volumes of this serum concentrated by polyethyleneglycol (14) to an incomplete anti-D titre of 512 were used to sensitize one volume of packed red cells Neither these

TABLE 3

Agglutination of Red Cells Sensitized by Various Anti D Sera

Anti D serum	Antiglobulin serum K 980: 64	128	256	512	1024	Anti antibody serum 141: 1	2	4	8	16	32	64	128
	Antiserum and reciprocal of dilutions												
SV 12/12 57	+++	++	++	+	—	+++	+++	+++	+++	++	++	+	—
SV 28/12 57	+++	+++	++	++		+++	+++	+++	+++	++	++	+	—
SV 11/8 64	+++	+++	++	+		+++	+++	+++	++	++	+	+	—
SV 18/1 65	+++	+++	++	+	—	+++	+++	+++	+++	++	++	+	—
3109	+++	++	++	+	—	++	++	++	+	—	—	—	—
3083	+++	++	++	+	—	+++	+++	+++	+++	++	++	++	—

Anti D serum	Anti Gm(f) serum VJ: 1	2	4	8	16	32	64	128	Anti Gm(b) serum 2535: 1	2	4	8	16	32	64	128
	Antiserum and reciprocal of dilutions															
SV 12/12 57	+++	+++	+++	++	++	+	—	—	+++	+++	+++	++	++	+	—	—
SV 28/12 57	+++	+++	+++	++	++	+	—	—	+++	+++	+++	++	++	++	—	—
SV 11/8 -64	+++	+++	+++	++	++	+	—	—	—	—	—	—	—	—	—	—
SV 18/1 65	+++	+++	+++	++	+	+	—	—	—	—	—	—	—	—	—	—
3109	—	—	—	—	—	—	—	—	+++	+++	+++	+++	+++	++	++	—
3083	+++	+++	+++	+++	++	++	+	—	—	—	—	—	—	—	—	—

Controls Unsensitized red cells and the respective antisera (1 1 to 1 512) no agglutination
Sensitized red cells and saline no agglutination

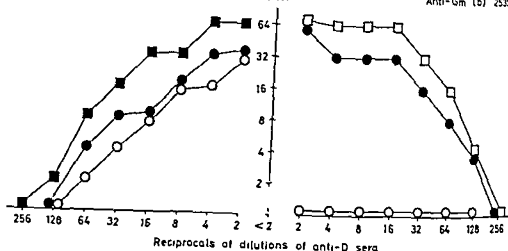


Fig 1
Titre of anti Gm(b) 2535 and anti Gm(f) AJ against red cells sensitized by anti D in serial twofold dilutions Titres in antiglobulin test for each row of sensitized red cells anti-D S V 1957 512, anti D S V 1965 256, anti D 3083 512 and anti D 3109 256 Symbols as in Fig 2

nor red cells which had been sensitized by mercaptoethanoltreated serum S V 1965 to destroy possible inhibitors, were agglutinated by anti Gm (b)

The influence of the amounts of anti-D in agglutination reactions was tested in two dimensional titrations Serum S V 1957 and 1965, and the 2 controls (3109 and 3083) were used in twofold dilutions for sensitizations of red cells, tested against anti-Gm (b), anti-Gm (f) and antiglobulin serum (Fig 1) The indirect antiglobulin titre of each row of sensitized red cells was higher than the titre obtained by specific *anti-Gm sera* Red cells sensitized with anti-D 3109 or -3083, agglutinated by only one of the anti-Gm sera (monospecific), gave the strongest reactions A weaker but principally similar reaction was obtained when the bispecific anti-D S V 1957 was used Anti-D S V 1965 did not detect anti-Gm (b) in agglutination reaction, while the reaction with anti-Gm (f) was similar to that with the other batches of sensitized red cells agglutinated by anti-Gm (f)

Absorption Experiments

Absorptions were performed with red cells sensitized by sera S V, -3109 and -3083 and 2 other Gm (a—x—b+f+) anti D sera (3066 and 3500) selected on the basis of the Gm type and the specificity in agglutination tests Anti-D 3066 detected only anti Gm (f), and anti D 3500 only anti-Gm (b) Red cells sensitized by the potent anti-D 3091 from a Gm (a+x+b—f—) donor, and red cells exposed to Gm (a—x—b+f+) serum 3092 containing no anti-Rh antibodies, were used for

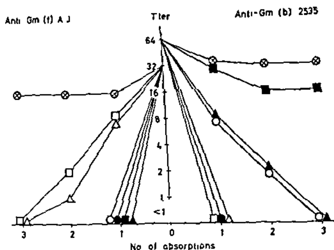


Fig 2

Absorption of anti Gm(b) 2535 and anti Gm(f) A.J by red cells sensitized by different anti D sera Each anti Gm serum diluted 1 2 was absorbed three times by an equal volume of packed sensitized red cells After each absorption the supernatant fluids were tested for activity Anti Gm(b) and anti Gm(f) activity was tested against red cells sensitized by anti D 3109 and 3083 respectively

control absorptions They gave very similar results Only the figures obtained with serum 3092 are recorded (Fig 2)

The sensitized red cells agglutinable by an anti Gm factor removed the factor completely in one absorption Red cells sensitized by some anti D sera were not agglutinated by, but did remove the respective anti Gm factor in repeated absorptions (Fig 2) Red cells sensitized by anti D S V 1965 or anti D 3066 removed anti Gm (b) 2535 in 3 absorptions, and gave a similar absorption of Snagg anti Gm (b) Aflatre and of the original anti Gm (b) J K No absorption occurred when anti D 3083 sensitized red cells were used

Red cells sensitized by anti-D 3109 or 3500 were agglutinated by anti Gm (b) only but absorbed anti Gm (f) in 3 absorptions

Consequently anti-D sensitized red cells not agglutinable by a certain anti Gm factor might contain a small proportion of anti D molecules of the homologous Gm type

Experiments with Eluted Anti-D.

Anti-D S.V. 1965, -3109, -3083 and -3091 selected on the basis of their Gm specificity in the agglutination and absorption tests above, were eluted from red cell stromata. Anti-D S V 1957 was excluded because of insufficient available serum As a substitute, the similar bispecific Gm (b) and Gm (f) anti-D 3417 was used Blood donor serum 3092 served as control. The eluates were titrated for anti D activity (Table 4). Red cells sensitized by eluted anti-D showed the same specificity in agglutination reactions as when the respective anti-D serum was used.

TABLE 4
Inhibition of Anti-Gm(b) by Eluted Anti-D Antibodies

Dilution of eluate	Anti-D and control sera with Gm type of donors					
	S V 1965	3417	3109	3083	3092	3091
		Gm (a— x— b+ f+)				Gm (a+x+b—f—)
1 1	—	—	—	—	—	+++
1 2	—	—	—	—	—	+++
1 4	+	—	—	++	++	+++
1 8	++	+	—	++	++	+++
1 16	+++	++	+	+++	+++	+++
1 32	+++	++	++	+++	+++	+++
1 64	+++	+++	+++	+++	+++	+++
Titre of eluate in indirect antiglobulin test	256	256	256	512	< 1	512
Specificity of anti-D sensitized red cells in agglutination test	Gm(f)	Gm(b) Gm(f)	Gm(b)	Gm(f)	—	Gm(a) Gm(x)

Test system Anti Gm(b) 2535 1 21 (3 agglutinating units) and O, R'r red cells sensitized with anti C 3360 +++
Controls Unsensitized red cells and the respective eluates (1 1 to 1 64) no agglutination Sensitized red cells and saline no agglutination No inhibition of agglutination in test system by eluate from saline treated stromata

Red cells (O,R'r) sensitized by incomplete anti C 3360 were used to determine the anti-Gm (b) inhibiting activity of the eluates As these red cells did not react with anti-D, the eluates could not influence the sensitization of the red cells The anti-C sensitized red cells were agglutinated by anti *Gm* (*b*) Three agglutinating units of anti-*Gm* (*b*) 2535 were used in the inhibition experiments The eluates were titrated with calibrated pipettes rinsing the pipette between each step The inhibition by eluates obtained with serum S V 1965 and serum 3083 was very similar to that of the control serum 3092, while anti-D 3417, and even more anti-D 3109, exhibited somewhat stronger inhibition (Table 4) Eluate obtained with serum 3092 which contained no anti-

body directed against O red cells showed some unspecific adsorption of gammaglobulins to the stromata Eluted anti-D 3091 from a Gm (a+x+b− f—) donor and eluate from salinetreated stromata did not inhibit even when undiluted eluate was tested against anti-Gm (b) in twofold dilutions

The anti Gm (b) *inhibiting capacity of* whole serum S V. was tested and showed no difference between the 1957 and 1965 samples

Anti Gm Activity

The samples of serum S V unabsorbed and absorbed of anti-D were *tested in the Waaler-Rose test and against red cells sensitized by* different anti D sera The titres in the *Waaler Rose* test were less than 8 Red cells sensitized by Gm (b) or Gm (f) anti-D 3109, -3083, -3417, S V 1957 or S V 1965, *respectively*, *were not* agglutinated by serum S V Red cells sensitized with Gm (a+x+) anti-D 3091, and anti-CD Ripley were agglutinated by serum S V 1964 and 1965 (Table 5) The agglutinating activity was equally strong in serum unabsorbed and absorbed of anti D activity, was completely inhibited by 1 per mille gammaglobulin and was destroyed by mercaptoethanol The two 1957 samples showed no agglutinating activity Two-dimensional agglutination inhibition experiment (7) showed that the agglutinating activity of serum S V was inhibited to a similar degree by Gm (a+x+) and Gm (a+x—) sera but not by Gm (a—x—) sera Accordingly serum S V from 1964 and 1965 contained anti Gm (a) Serum from the husband and *first child both Gm* (a+x—), *inhibited as the Gm* (a+) controls Serum from the third child, Gm (a—x—), did not inhibit Serum from the second child was not available when the inhibition experiments were performed

TABLE 5
Agglutination of Anti D Sensitized Red Cells by Serum S V

	Red cells sensitized with										
Dilution of	anti D 3091					anti CD Ripley					
serum S V 1	1	2	4	8	16	1	2	4	8	16	32
S V 12/12 57 in saline	—	—	—	—	—	—	—	—	—	—	—
S V 28/12 57 in saline	—	—	—	—	—	—	—	—	—	—	—
S V 11/8 64 in saline	+++	++	++	+	—	+++	+++	+++	++	+	—
1‰ γ glob	—	—	—	—	—	—	—	—	—	—	—
S V 18/1 65 in saline	+++	+++	++	+	—	+++	+++	+++	++	+	—
1‰ γ glob	—	—	—	—	—	—	—	—	—	—	—

Controls Unsensitized red cells and the respective samples of serum S V (1 1 to 1 32) no agglutination Sensitized red cells and saline no agglutination

DISCUSSION

All the serum samples labelled S V corresponded in Gm, Hp and Gc types and contained anti-B but not anti-A. The frequencies of the involved phenotypes are Gm (a—b+): 39 37 per cent (10), Gc (2—2) 7 per cent (23), Hp (2—2). 40 6 per cent (4), and of blood group A 49 37 per cent (12, p 61). The probability that two different persons should have this combination of phenotypes by chance is p = 0 0055 This gives high statistical significance that all the tubes really contained serum from the same individual

The experiments showed that a change in proportion of Gm (b) and Gm (f) genetic determinants had occurred in the anti-D antibody S V from 1957 to 1964 No change in antibody specificity was detected, but a slight decrease in the titre was found

No inhibiting activity of serum S V could explain the depletion of Gm (b) anti-D molecules Anti-Gm (b) was not detected, and treatment by mercaptoethanol to destroy possible inhibitors did not change the specificity Eluted anti-D S V 1965 did not inhibit anti-Gm (b) more than the controls, and red cells sensitized with the eluates or with the original anti-D serum showed a similar specificity The total anti Gm (b) inhibiting capacity of whole serum S V was not altered This indicated that the difference in proportion of Gm (b) and Gm (f) genetic determinants of anti-D S V in 1957 and 1965 really reflected a decrease in the production of Gm (b) anti-D compared to Gm (f) anti-D

Disappearance of the factor Gm (a) from whole serum of patients with acute myeloblast leukaemia has recently been described (26) However a change in Gm character of an isolated antibody in a healthy individual has not previously been observed

One cell clone is probably committed to produce only one kind of antibody (20) and "monoclonal proteins" did not contain more than one of the gene products Gm (b) and Gm (f) (18) In serum S V 1957 the proportion of Gm (b) to Gm (f) anti-D molecules was about 1 1 shown by similar activity in test with anti-Gm (b) and anti Gm (f) (Table 3) In serum S V 1965 the amount of Gm (b) anti-D molecules was too small to be detected by agglutination However red cells sensitized by this anti-D were still able to absorb anti-Gm (b)

Other experiments showed that the proportion of Gm (b) to Gm (f) molecules on red cells sensitized by sera similar to serum S V 1965 is about 1.8—1 16 (to be published) The outstanding difference in proportion of the two genetic characters in anti-D from 1957 and 1965 gives evidence that the two characters belong to different molecules produced by independently working cell clones

The Gm factors are localized to the γG-globulin heavy chains with their recently detected subgroups The Gm (b) factor belongs to the V_I subgroup and the Gm (a) and Gm (f) characters to the We subgroup

(1a) Using the Gm factors as markers the present finding demonstrated a change in heavy chain subgroups of anti D S V

The depletion in production of Gm (b) anti D molecules in serum S V can be compared with observations on anti D antibodies and myeloma proteins indicating that only a minor part of antibodies contain Gm (b) while a major proportion carry the Gm (a) or Gm (f) factors (11 17, 18) *Martensson* (18) confirmed this showing that isolated myeloma proteins of type Gm (b+f—) were relatively much more inhibiting than those of type Gm (b—f+) compared to the respective inhibiting capacity of the donors whole serum The present finding that the production of Gm (f) anti D molecules has been well maintained while the Gm (b) type has decreased significantly, may point to a possible different ability of the two types of cell clones involved to maintain antibody production

Red cells sensitized by some anti D sera absorbed, but were not agglutinated by the corresponding anti Gm factor, demonstrating that absorption is a more sensitive method for the detection of small amounts of anti D molecules of a certain Gm specificity

The number of D receptors on the Rh positive red cells is relatively scanty (22 p 153) When two genetic types of anti D molecules have to share the antigenic sites the Gm type in depletion may lose its ability to detect the corresponding anti Gm factor

In this field other absorbing but non agglutinating systems are known Horse erythrocytes sensitized by rabbit antibody were not agglutinated by but did absorb rheumatoid factor (21) Recently, incomplete rheumatoid factors were detected in the *Waaler Rose* test by indirect antiglobulin technique (28) Possibly anti Gm factors absorbed by anti D sensitized red cells without causing agglutination can be detected by specific anti-γM globulin sera

The anti Gm (a) factor in the serum S V and the change in Gm specificity of the anti D appeared at the same time Judged from the loss of activity by mercaptoethanoltreatment anti Gm (a) was of the γM globulin type (6) Isospecific anti Gm factors have been described in polytransfused patients (1) *Fudenberg* (5) described anti Gm (a) resulting from foeto maternal immunization In the family of S V (Table 1) the possibility of immunization of the Gm (a—) mother with Gm (a+) globulins from father or child no 1 existed Children nos 2 and 3 were Gm (a—) It is unlikely that an immunization from child no 1 born in *August* 1956 should not be detectable by antibody production until after *December* 1957, and no other antigenic stimulus is known

Immunization through the sperms of her husband remained a possibility Gm (a) antigens have been found in sperms of Gm (a+) men who were ABO secretors (13) Inhibition experiments with sperms from the husband of S V were inconclusive The material was scanty and probably unsatisfactory due to long transport

SUMMARY

1 One incomplete anti-D antibody (S V) was found in which a change in proportion of Gm (b) and Gm (f) molecules had taken place from 1957 to 1964 These findings indicated a change in the *production* of heavy *chain* subgroups of anti-D γG globulin

2 Serum S V 1964 and 1965 and four other sera contained highly different quantities of Gm (b) and Gm (f) anti-D molecules Only one of the anti-D antibodies was Gm (b−f+) and no Gm (b+f−) anti D was found Anti-D S V 1957 contained about equal amounts of Gm (b) and Gm (f) molecules The importance of performing absorption experiments in order to detect minor proportions of certain genetic types of isolated anti-D antibodies is stressed

3 In serum S V an isospecific anti-Gm (a) factor had developed from 1957 to 1964 The antigenic stimulus is uncertain

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BRIEF REPORT

EARLY GROWTH OF THE BERGEN A4 ASCITES CARCINOMA IN FEMALE MICE

By F. Hartveit[1]

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This idea was tested by injecting, intraperitoneally, 0.05 ml of whole tumour ascites from a 7-day transplant of the Bergen A4 ascites carcinoma that grows progressively in mice of this closed colony (4), into virgin female mice (16–17 weeks old) of this closed colony. Two groups were set up. One consisted of 7 mice in which the vaginal lavage specimen contained mucus, the other of 15 mice without mucus. The mice were killed 48 hrs later and the total packed cell volume (PCV) of the tumour in the ascites determined (5).

The results were as follows:

Mice with mucus – mean PCV of tumour 0.19 ± 0.04 ml
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This difference in means is statistically significant ($0.01 > P > 0.001$). The admixture of non tumour cells was similar in both groups.

The mechanism of this potentiation of tumour growth can not be elucidated from this experiment. But as it has previously been shown that the presence of mucus in these mice is indicative of prooestrus (1) it is likely that it is associated with the increased vascularity of the host tissues at this time. The present finding therefore suggests that *standardisation with regard to the phase of the oestrus cycle should be considered when setting up groups of female mice for studies concerning early ascitic tumour growth.*

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The Cancer Research Institute (Head Prof J Bichel M D) and the Clinical Department (Head Prof S Kaae M D) The Radium Centre Arhus C, Denmark

ON THE EFFECT OF ERYTHROPOIETIN AS AN ACCELERANT OF TUMOURGROWTH

By

E B Thorling

Received 28 iv 65

Erythropoietin is known as the factor(s), which given for longer periods to a normal person or most warm blooded laboratory animals will bring about a rise in the total circulating erythrocyte volume, when given in doses exceeding the normal production of the recipients The factor (Hormone) is produced by some action of the kidney, not yet fully known as a response to deficient oxygen supply to part of the kidney, most likely cells provided from the secondary capillary network Consequently high concentrations in serum can be expected in anaemia, uncompensated high altitude conditions", local circulatory failure in the kidney and as a result of the function of various toxic agents *e g* Cobalt Carbonmonoxide etc

Erythropoietin has been concentrated from serum and urine from man and laboratory animals in many laboratories and the biological effect of the hormone is not species specific Partially purified preparations have been characterized as alfa2 globulin(s) with a high content of carbohydrates

Erythropoietin stimulates the erythropoiesis in the bone marrow (and extramedullary), and in most laboratories this has been found to be the only action of the hormone Thrombopoiesis and granulopoiesis are not significantly influenced by partially purified preparations of the hormone (Review, *Remmele* 1963)

Whether other organs might be affected by erythropoietin is not known as it has not been thoroughly looked for

The investigations by *Leaders et al* (1962) therefore are of great interest This group found that erythropoietin active preparations from two different laboratories had a stimulating effect on the growth of the Novikoff Hepatoma Later the same group investigated the effect of erythropoietin in tissue cultures and was able to demonstrate a similar effect on two strains of cells

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This preparation is referred to as *an ser rab* The erythropoietin content of this ... 0 5 cobalt units/ml

units ml The activity was not tested in our own laboratory but the mice in series 11 developed a significant raise in the reticulocyte count after injections with the solution

11 series of experiments were carried out

1 *C3H mice* 2 groups of each 10 mice On 4 consecutive days 0 1 ml of fraction A was given subcutaneously On the 4th day 10^6 PBH tumourcells were transplanted subcutaneously The control group received saline instead of fraction A

2 *C3H mice* A repetition of 1

3 *C3H mice* Analogous to the first 2 series, but 0 5 ml × 4 fraction A was given instead of 0 1 ml × 4

4 *AK/A mice* Analogous to 3 using the JB1 tumour

5 *Balb C mice* Analogous to 3 and 4 using the plasmocytoma

6 *C3H mice PBH tumour* To demonstrate whether a possible stimulating effect acted on the host or on the tumour cells one half of a tumourcellsuspension (10 ml) was incubated with 2 ml of fraction A for 2 hrs at 37° C while the other half was incubated in saline alone After incubation the cells were washed and resuspended before transplantation 3 6 × 10^6 cells were given to each mouse

7 *Balb C mice* 4 groups of each 10 mice Each mouse was transplanted with 3 5 × 10^6 plasmocytomacells subcutaneously on the thoraxwall Every second day after the transplantation for 3 weeks the following preparations were given

group 1 0 5 ml of *saline*
group 2 0 5 ml of fraction A
group 3 0 5 ml of an ser rab
group 4 0 5 ml of nor ser rab

8 *C3H mice HB tumour* 4 groups of each 15 mice The erythropoietin preparation used in this and the next series was prepared from urine from another patient with aplastic anaemia A dry powder was prepared according to the procedure

* The preparation was kindly sent to me from Dr *P Hansen*, Statens Seruminsitut, Copenhagen

If this tumour growth stimulating effect of erythropoietin is not confined to the Novikoff Hepatoma but is a more general feature, the clinical consequences of these facts would be of great extent. In anaemic patients with malignant diseases the erythropoietin content in the serum is often very high (Review of the leucosis, *E. B. Thorling* 1965). The erythropoietin content in serum can be normalized by transfusion of blood to normal haematocrit values. If therefore erythropoietin stimulates the malignant growth, these patients should more carefully be kept at normal haemoglobin values. We therefore found it imperative to investigate further this possible growth stimulating effect of erythropoietin on various transplantable mousetumours.

MATERIALS AND METHODS

Three strains of mice were used.

C_3H/A mice. Maintained in the institute (The Radium Centre for Jutland, Århus) since 1950 by brother-sister inbreeding.

AK/A mice. Maintained in the institute by brother-sister inbreeding since 1950.

Balb/C albino mice. Received from the Chester Beatty Research Institute in London Febr. 1963 and maintained in the institute only for a few generations.

The tumours transplanted to the respective strains were as follows.

In the C_3H strain. The PBH tumour: originally (1951) a highly differentiated *mamma adenocarcinoma* which arose spontaneously in the C_3H strain. Tumour later became more anaplastic and has now been carried through nearly 300 generations (*K. Nielsen* 1962).

Another *mamma adenocarcinoma* was used in the last series 8-11. This *tumour HB* is less dedifferentiated than the PBH tumour. As the former it arose as a spontaneous tumour in the C_3H strain in our institute about 5 years ago.

In the AK/A strain. The JB1 tumour. Described as a plasmocytoma by *J. Bichel* 1951 who now considers the tumour to be a small celled sarcoma with many plasmacells.

*In the Balb/C strain. A plasmocytoma** which arose spontaneously in the strain. This *plasmocytoma* is characterized by the appearance of typical myelomaproteins in the serum.

All the tumours were transplanted subcutaneously on the thorax wall to mice about 2 months old by cell suspensions in saline.

To be sure of comparability the transplantations were performed in the following sequence:

Control animal—animal given the erythropoietin preparation—control animal and so on. –

The tumourgrowth was registrated every second day (the length and width in mm's at right angles) and the survival time was noted.

Autopsy. Preparations for microscopic studies were made from the tumour, liver, spleen, kidneys and thoraxorgans.

Erythropoietinactive preparations.

Source. Urine from patients with aplastic anaemia. Serum from rabbits made anaemic by bleeding. Serum from sheep made anaemic by injections of phenylhydrazine.

CONCENTRATION PROCEDURE FOR URINARY ERYTHROPOIETIN

The urine is collected in a deep freezer at —20° C. At preparation it is tawed and filtered 0.1 N ... acid Kaolin (Fisher K 5) is added 1 % ... 4° C for 24 hrs. Centrifugation is t... arged.

* The tumour was kindly supplied to us by the Chester Beatty Research Institute in London.

The precipitate is washed by pure ethanol and dried in vacuum (case-
8 and 9)

by bleeding was not con-
serum was acidified by
Most albumin is hereby
was dialysed against de
precipitated After centri-
fugation the supernatant is equilibrated against saline for 24 hrs at 4° C.

This preparation is referred to as *an ser rab* The erythropoietin content of this solution was determined to *0.5 cobalt units/ml*

units ml The activity was not tested in our own laboratory but the mice in series 11 developed a significant raise in the reticulocyte count after injections with the solution

11 series of experiments were carried out

1 *C_3H mice* 2 groups of each 10 mice On 4 consecutive days 0 1 ml of fraction A was given subcutaneously On the 4th day 10^6 PBH tumourcells were transplanted subcutaneously The control group received saline instead of fraction A
2 *C_3H mice* A repetition of 1
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4 *A.K A mice* Analogous to 3 using the JB1 tumour
5 *Balb C mice* Analogous to 3 and 4 using the plasmocytoma
6 *C_3H mice PBH tumour* To demonstrate whether a possible stimulating effect acted on the host or on the tumour cells, one half of a tumourcellsuspension (10 ml) was incubated with 2 ml of fraction A for 2 hrs at 37° C while the other half was incubated in saline alone After incubation the cells were washed and resuspended before transplantation 3 6 × 10^6 cells were given to each mouse
7 *Balb C mice* 4 groups of each 10 mice Each mouse was transplanted with 3 5 × 10^6 plasmocytomacells subcutaneously on the thoraxwall Every second day after the transplantation for 3 weeks the following preparations were given

group 1. 0.5 ml of saline
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8 *C_3H mice HB tumour* 4 groups of each 15 mice The erythropoietin preparation used in this and the next series was prepared from urine from another patient with aplastic anaemia A dry powder was prepared according to the procedure

* The preparation was kindly sent to me from Dr *P Hansen* ,Statens Seruminsitut, Copenhagen

just described A solution was made containing 1 mg/ml The erythropoietin activity was estimated to *0 75 cobalt units/ml*

0 5 ml of the following preparations were given daily for 8 days On the 4th day the mice were transplanted with 2 × 10^6 HB tumourcells

group I	Saline only	
group II	Erythropoietin preparation	before transpl
	saline	after
group III	*Saline*	*before*
	Erythropoietin preparation	*after*
group IV	1 mg of Cryst Human Albumin	before
	saline	*after*

9 *C3H mice HB tumour* 2 group of each paration was used as in series 8 but in for 4 days, transplanting on the 4th The [illegible] doses

10 *C3H mice HB tumour* 2 groups of each 15 mice One group was placed in a decompression chamber *for* 3 weeks at 0 5 Atm s pressure *16 hours* a day After one week at ambient pressure this group and the control group were trans *plantated with 2 × 10^6 HB tumourcells On the day of transplantation the* mean haematocrit value in the treated group was 75 8 per cent compared to the con trol group *in which it was 52 3 per cent The reticulocyte count in the treated* group was approximately zero indicating a depressed erythropoiesis presumably caused by an abolished erythropoietin production

11 *C3H mice HB tumour* 2 groups of each 30 mice ERS3 plasma from phenyl hydrazine treated sheep was used 1 ml containing 1 7 Standard B units was given daily for 4 days, the transplantation carried out on the 4th day 2 hours after the injection of erythropoietin A control group received saline in equal doses

All preparations were given subcutaneously in the opposite side of the trans plantation

RESULTS

The first 2 series (C3H mice, PBH tumour) gave suspicion of a differ ence in growth rate of tumour between the treated group and the con trol group The difference could nevertheless not be proved significant nor could any difference in survival time be demonstrated

In the next series (3) the erythropoietin dose was multiplied by five, using the same strain and the same tumour It was now obvious that the treated group had a faster tumour growth than the controls In the interval from about the 4th to 10th day after the first appearance of *palpable tumours the difference in tumour size could* be demonstrated to be highly significant using the Students 't' test In the succeeding days the difference in tumour size diminished and could no *longer be* shown *to* be *significant* The survival time in the 2 groups was the same

The same dose of erythropoietin was used in series 4 *Similar results* were found indicating that the JBI tumour grows faster in the treated *AK/A* mice than in the controls As found in series 3 this difference which for a short *interval* could be *proved significant*, diminished again and no difference in the survival time was observed

Incubation of PBH cells for 2 hs at 37° C in erythropoietin *rich saline* did *not* alter the behavior of the tumour which arose after transplan- tation of the tumourcells

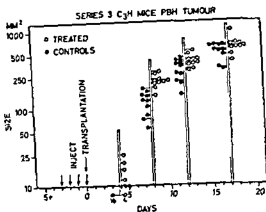


Fig 1

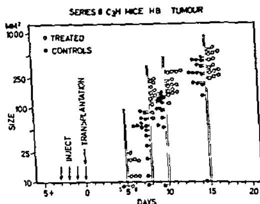


Fig 2

Figs 1 and 2

The diagrams demonstrate the growth of the two different mamma carcinomas in series 3 and 8 It is clearly seen that the difference between the treated group and the control group vanishes as the growth progresses

In the Balb/C strain transplanted with the plasmocytoma no effect on the tumour could be ascribed to erythropoietin In this strain the erythropoietin was given (series 5) first before the transplantation and second (series 7) every second day after the transplantation for 3 weeks Furthermore, a preparation from serum of anaemic rabbits was used all with no significant effect on the growth of the tumour

In the two next series 8 and 9, using the HB mamma adenocarcinoma, the effect on the tumour growth could again be demonstrated This seems to be important as the erythropoietin preparation used was prepared from another patient and in another way than in the first 7 series When the erythropoietin preparation was given in the days after the transplantation the effect was less than when given in the days be-

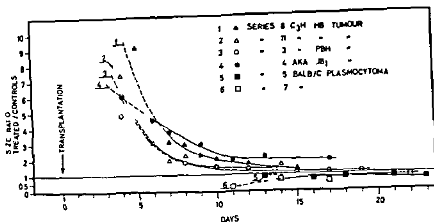


Fig 4

fore and on the day of transplantation No significant effect could be attributed to human albumin (1 mg × 4) which is the most abundant contaminant in the preparation

The difference in survival time between the group treated with erythropoietin before transplantation and the control group (50 per cent dead) was 5 days, which is not significant

In series 9 the difference between the treated and the control group was still more obvious This group received a larger dose of erythropoietin *and the tumourcell dose was chosen so little that only two* animals in the control group took the transplantation The take in the treated group was 7 out of 10 The size of the tumours are demonstrated on the photographs taken *19 days* after the transplantation

In series 10 no significant difference was found neither in the growth rate of the tumour nor in the survival time, thus induced polycythemia and supposed low erythropoietin production had no significant restrictive influence on the tumour growth

In series 11 the effect on the tumour growth rate could again be demonstrated The erythropoietin active preparation was here prepared from plasma of sheep and is in most respects different from the preparations made from urine from patients with aplastic anaemia The

Fig 3

Ph to cf the mice in series 9 19 days after transplantation
Right row Mice given erythropoietinactive preparations
Left r w C ntrol mice

effect of the various different preparations is very similar which is most clearly seen in Fig 4.

In these series weight was controlled, and the animals did not lose weight in relation to the injections

In none of the series any significant difference in the survival time between treated groups and control groups was observed

DISCUSSION

The problem of a possible tumour growth stimulating effect of erythropoietin is a question of the specificity of this principle

The erythropoietin is produced when part of the "erythrocytorgan" is removed from the organism, and is in many respects analogous to the factors produced in partial hepatectomy or unilateral nephrotomy In these cases factors are produced inducing regeneration of the respective organ Only a few works are concerned with the specificity of these factors *Paschkis* found that partial hepatectomy resulted not only in regeneration of the liver which has been known since 1890 (*Ponfick*) but that also the mitotic activity of the corneaepithelium increased He could furthermore demonstrate that the contralateral hypertrophy after unilateral nephrectomy was accelerated when part of the liver was removed In hypophysectomized rats he found that the width of the epiphyseal line of the tibia was greater in animals submitted to partial hepatectomy than in the hypophysectomized controls Many investigations support the suggestion that the regenerating liver produces a factor of humoral nature which is responsible for the effect It has been possible to transfer the effect between parabias, one of which was submitted to partial hepatectomy *Wenneker & Sussman, Bucher et al* The factor has been transferred from a partially hepatectomized animal to normal animals *by serum, Friedrich-Freska & Zaki* Reviewed by *Paschkis* (1958)

Leaders et al found 1962 an elevated Fe 59 uptake in the erythrocytes after partial hepatectomy in rats indicating a stimulation of the production of erythrocytes, probably caused by the livergrowth stimulating factor This is not yet proved to be an effect of a special livergrowth factor for even under normal conditions probably 10 per cent of the erythropoietin is elaborated in the liver (*J W Fisher et al, Z Kuratowska*)

In some instances partial hepatectomy could be shown to accelerate the growth of transplanted tumours, *Paschkis, Trotter, Leaders*, most often hepatomas but also a mammary carcinoma (Walker 256 tumours)

The livergrowth stimulating factor(s) is consequently not organ specific, but seems to exert its effect on various organs

Similar growth promoting factors of probable humoral nature, produced in fast growing tissues *e g*, malignant tumours are known

The presence of a mouse mammary cancer accelerant factor *in ex-*

tracts from fast growing mammary tumours in mice is well established by *H H Shear et al*, *C Martinez et al*, *G Miroff et al* Accelerated growth indicated by gain in weight and raised mitotic activity in the liver is described by *E Annau* to occur in rats transplanted with a reticulosarcoma and in mice transplanted with *3 different tumours* 2 mammary carcinomas and 1 fibrosarcoma Similar works were carried

ie spleen in mice transplanted

Extracts from mouse mammary carcinomas *could be shown to stimulate* the growth of lymphnodes in the recipient mouse, *S Albert B F Argyris* found that the ductus epithelium of the mammary gland revealed an increased mitotic activity and augmented growth in animals transplanted with Ehrlich ascitestumour and later he demonstrated a stimulated growth of the epidermis over *subcutaneously transplanted* Ehrlich ascitestumours

H Browning made some investigations in which he simultaneously transplanted to the anterior chamber of the mouse eye normal and malignant tissues He could demonstrate that the normal tissue grew faster when malignant tissue was present than when *the normal transplantate* was alone in the eye chamber He attributes this effect to some factors produced by the tumourcells

Not only tumour tissues but also other fast growing tissues have been investigated in this respect *Charlotte A Schneyer* showed that *tumour transplantates inoculated with embryonic cells grew faster* than the transplantate itself (Adult cells had the opposite effect) She attributes this effect to some substance produced by the embryonic cells In this connection the investigations by *K Blomquist* should also be mentioned She found that intraperitoneal injection of extracts from regenerating liver *obtained from rats submitted to partial hepatectomy* produced malignant growth in the recipient rat in 75 per cent of the cases, whereas injections of the analogous preparation from the liver tissue removed at the partial hepatectomy had no such effect

Concerning the erythropoiesis a number of tumours coexisting with erythrocytosis are described in man and in certain investigations erythropoiesis stimulating factors have been demonstrated in extracts of the tumours lately *P Hansen, E B Thorling, P Boivin* Reviewed by *R R Donati W Remmele*

When the unspecificity of the stimulating effect in the investigations just referred to is considered it would not be surprising if the erythropoietin at least in some instances might stimulate the growth of certain tumours

In *Leaders* first work on this subject there seems to be a stimulating effect on the growth of the Novikoff Hepatoma Leaders used two preparations made from serum of rabbits and sheep, respectively The animals were rendered anaemic by injections of phenylhydrazine This

drug is known to induce a haemolytic anaemia, but also to damage the liver As the erythropoietin preparations used are not pure, they may very well have contained a liver-growth promoting factor produced in the liver of the donor animals If this is the case it is not surprising that the hepatoma which is rather highly differentiated (*Novikoff*) would respond to this factor Furthermore, Leaders could demonstrate that partial hepatectomy stimulated the growth of the tumour Cobalt was given to the transplanted animals as cobalt is known to stimulate the production of erythropoietin The results in this last assay are not too convincing In later works *Leaders* investigated the effect of erythropoietin active preparations in tissue cultures Under certain circumstances he was able to demonstrate an effect on the growth rate of the cultures, in others he was not

W McFarland et al (1964) were unable to demonstrate any effect on human leucocytes in tissue cultures

In my own investigations mentioned above it was not possible to find any significant differences in survival time between the treated and untreated animals A significant difference could be demonstrated in the tumour growth in the C3H strain transplanted with the 2 different mammary carcinomas and in the AK/A strain transplanted with the JB1 sarcoma

Although there are some signs of an unspecific growth stimulating effect of erythropoietin the specificity of the factor is rather more obvious

SUMMARY

11 series of experiments have been carried out to demonstrate whether erythropoietin active preparations might act as stimulation for the growth of malignant tumours in mice 4 tumours were investigated in 3 strains of mice A growth stimulation could be demonstrated on two mammary adenocarcinomas in the C3H strain and on an aplastic sarcoma in the AK/A strain whereas no effect was found on a plasmocytoma in the Balb/C strain The effect found was an earlier appearance of the tumour and a faster growth in the groups given the erythropoietin active preparations than in the control groups The erythropoietin preparations were usually given for 4 days, the transplantation being carried out on the 4th The effect seems to vanish as the tumour develops and the difference in survival time, which was regularly present, could not be proved significant

Only a weak effect was observed in these experiments and although the effect could be reproduced using 3 different erythropoietin active preparations, none of these were pure erythropoietin, and consequently further research is required to establish that erythropoietin is responsible fo the effect observed

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CHRONIC TOXICITY OF DIETARY SODIUM MONOFLUOROPHOSPHATE IN GROWING RATS, WITH SPECIAL REFERENCE TO KIDNEY CHANGES

By

HEMMING POULSEN and YNGVE ERICSSON

Received 17 v 65

Sodium monofluorophosphate, Na_2PO_3F, is a fluorine compound which has attracted great interest in later years, especially in connection with the utilization of fluorine for caries prevention Reviews and original investigations of its properties of relevance for this utilization have been given in papers by *Ericsson* (1961 a, b, 1963 a, b) and *Ericsson, Ullberg & Santesson* (1961) Among these properties is that of its low acute peroral toxicity as reported by *Shourie, Hein & Hodge* (1950) and *Smith et al* (1960)

In an autoradiographic and quantitative investigation on the distribution of F^{18}, P^{32}-labelled Na_2PO_3F in the animal body *Ericsson & Hammarstrom* (1965) found notable concentrations of F^{18} in the liver and the spleen following the administration as PO_3F ion in contrast to the administration as simple fluoride ion This raised the question *whether the PO_3F ion would exert special toxic effects on the liver or a* chronic toxicity that was high in comparison to its acute toxicity The present investigation was designed to answer this question

MATERIAL AND METHODS

Male white rats weighing about 100 grams at the start of the experiments were used in this study They were raised in a standardized way by the Anticimex Co Stockholm on pellets containing about 28 ppm fluoride and tap water containing about 0 1 ppm fluoride

In a four week pilot study 20 rats were divided in 5 groups of four animals each for the preliminary testing of two concentrations of Na_2PO_3F in comparison with two concentrations of NaF and one control group Every animal was ear marked and kept in a separate cage and was given *ad libitum* a diet of the following basic composition

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Our thanks are due to Professor *Håkan Rydin* Head of the Biological Department of the State Pharmaceutical Laboratory Stockholm for placing the resources of his animal laboratory at our disposal

This investigation was supported in part by *Research Grant DE 01654-02 from the National Institute of Dental Research National Institutes of Health United States Public Health Service*

Skim milk powder	32 3 per cent
Potato flour	41 2 per cent
Dried yeast	8 1 per cent
Arachis oil	2 8 per cent
Cod liver oil	13 6 per cent

This diet was the same as had previously been used in fluoride toxicity studies by *Pindborg* (1957 a b) with the *exception that* the shark liver oil used by this author was replaced by the fiftyfold quantity of cod liver oil with a corresponding reduction of the *arachis oil* This quantity of cod liver oil was calculated to give about the same vitamin D *supply* as the shark liver oil which was not available

For the different groups analytically pure fluorides were homogeneously admixed to the basic diet to the following percentages

Group 1	0 17 per cent Na_2PO_3F
Group 2	0 34 per cent Na_2PO_3F
Group 3	0 05 per cent NaF
Group 4	0 1 per cent NaF
Group 5	no addition (control)

0 17 and 0 34 per cent Na_2PO_3F cent NaF respectively (about 226 the rat diet was found by *Pindbo* (1959) to cause partly reversible damage to the kidneys

The animals were *given tap water* containing about 0 1 ppm F *ad libitum*

The animals were weighed at the start and on the same day every following week of the experiment

After four weeks the animals were deeply anaesthetized with ether and decapitated *Liver spleen* and kidneys with adrenals were placed in 10 per cent neutral formalin in plastic *capsules* which were allotted code numbers The formalin solution was changed the following day

The author Y F who conducted the described experimental procedures in Stockholm then sent the specimens to the author H P in Copenhagen who carried out the pathological *examination* as a blind test The organs were embedded in paraffin and sections were *stained with* either haematoxylin and eosin van Gieson's connective tissue stain or PAS

After the pilot study had demonstrated that the selected fluoride *concentrations* were suitable for tests on the chronic toxicity (see Results) the main study was carried out in *principally* the same way but with the following changes

20 animals in each of groups 1 and 2
12 animals in each of groups 3 and 4
8 animals in group 5

One fourth of the animals in each group were to be sacrificed at each *of the* following *times* if surviving 4 8 12 and 16 weeks the basic diet was the same as that used by *Pindborg* et containing 16 2 per cent arachis oil and 0 27 per cent *halibut* liver oil

RESULTS

The weight curves of the animals in the pilot study appear in Fig 1 Groups 1–4 differed significantly from the control group after 4 weeks ($P<0\ 01$) while the difference between groups given the same quantity of fluorine as F or PO_3F ions was non-significant ($P>0\ 05$) The animals in groups 2 and 4 which received the highest *fluoride* concentrations had a yellowish, shaggy fur and skin changes *around* the eyes resembling those seen in some vitamin deficiencies

The weight curves of the animals surviving for 8 weeks in the main study appear from Fig 2 At 8 weeks group 3 (0 05 per cent NaF) differed significantly in weight from groups 5 and 1 (control and 0 17

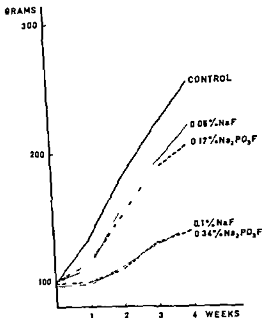


Fig 1
Weight curves of rats in pilot study

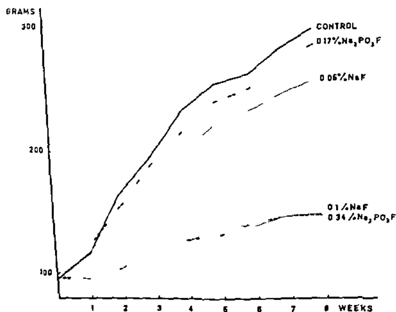


Fig 2
Weight curves of rats in main study that were sacrificed after eight weeks or more

per cent Na_2PO_3F) at the 0 01 P level, while the difference between the two last-mentioned groups was *non-significant* The animals in the two groups receiving the highest concentrations of Na_2PO_3F and NaF, respectively, had the same appearance as the corresponding animals in the *pilot study*, and two rats in the 0 34 per cent Na_2PO_3F group and three rats in the 0 1 per cent NaF group died before the end of the experiment

The pathological examination of liver, spleen and adrenals in the pilot study and in the main study showed no alterations in any of the groups In two rats given fluoride a very slight, focal proliferation of Kupffer cells was found, but the same phenomenon was observed in one of the control rats

TABLE 1

The Degree of Histological Kidney Changes in Rats Given 0 17 per Cent and 0 34 per Cent Na_2PO_3F and 0 05 per Cent and 0 1 per Cent NaF for 4, 8 12 and 16 Weeks (Rats Dead during the Experiment not Included)

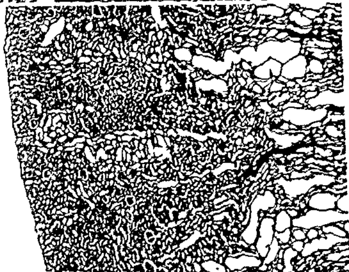
Group	Number of rats: Without kidney changes	Moderate kidney changes	Severe kidney changes	Total
1 (0 17% Na_2PO_3F)	8	13	3	24
2 (0 34% Na_2PO_3F)		1	21	22
3 (0 05% NaF)	7	2	7	16
4 (0 1% NaF)			13	13
5 (Controls)	12			12

In contrast, as shown in Table 1, the kidneys were abnormal in the majority of the animals given fluorides Also evident in Table 1 is the fact that the kidney changes were more severe in *groups 2 and 4* than in groups 1 and 3 i e the highest doses gave the most severe changes

The character of the histological changes was the same following NaF or Na_2PO_3F administration and the picture described by *Pindborg* (1950, 1957 a) and *Lindemann, Pindborg & Poulsen* (1959) was reproduced In *addition*, other observations were made which *will* be described in more detail

The most obvious alteration was, as described previously, dilatation of the Henle loops, most pronounced in the outer part of the medulla and the inner part of the cortex (Fig 3) It is striking that the dilatation in the four-week experiments ceased in the area where the tubule lays in close relation to the glomerulus (Fig 4) even though no occlusions or casts were found in this part of the nephron There was flattening of the epithelium in the dilated ducts and often it seemed to be absent

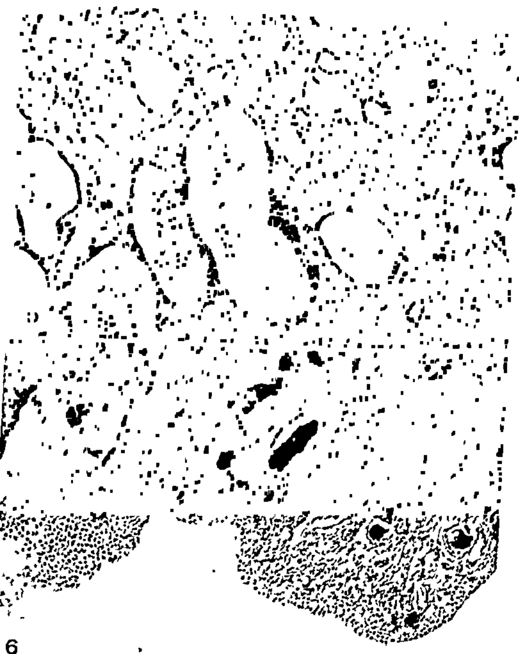




Figs 3–4

Fig 3 ney from a rat given 0 17 per cent *of the tubules is most pronounced* E stain × 67

Fig 4 a rat given 0 17 per cent Na_2PO_3F on of the tubules at the medullo- ... some of the tubules in the cortex this dilatation extends only as far as the glomeruli H and E stain × 52



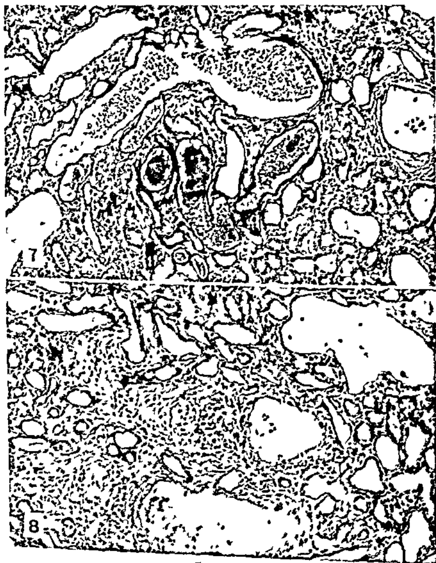
Figs 5 6

Fig 5 Dilated tubules with flattened epithelium and with some inflammatory cells in the lumen From a rat given 0 34 per cent Na_2PO_3F for four weeks *H and E* stain × 210

Fig 6 Dilated ducts with PAS positive casts in the apex of a pyramid from a rat given 0 34 per cent Na_2PO_3F for twelve weeks PAS stain × 210

(Fig 5) In association with this there was some infiltration of inflammatory cells in the surrounding interstitial tissue

In the more longstanding experiments the dilatation of the ducts was more severe and to some extent spread to other parts of the nephron,



Figs 7 8

Fig 7 Dilated tubules with PAS positive casts in the medullo cortical junction from a rat given 0 17 per cent Na_2PO_3F for twelve weeks PAS stain × 210

Fig 8 Granuloma in the medullo cortical area from a rat given 0.34 per cent Na_2PO_3F for twelve weeks H and E stain × 210

i e tubuli contorti II, tubuli contorti I and the collecting tubules In many dilated ducts, PAS positive casts were seen, some of which were associated with granulocytes, lymphocytes, plasma cells and macro phages The majority of the casts were located in the apices of the pyramide (Fig 6) and the medullo-cortical junction (Fig 7) Inter-



Figs 9 10

Fig 9 Granuloma in the inner part of the cortex from the same rat as in Fig 8 H and E stain × 210

Fig 10 Granuloma with slight infiltration of lymphocytes and with a few plasma cells in the periphery From the medullo cortical zone of a rat given 0 34 per cent Na_2PO_3I for eight weeks H and E stain × 525

stitial inflammation was more pronounced, and after 12 and 16 weeks an increasing fibrosis was found During the first four weeks all glomeruli were normal, but in rats with fibrosis of the kidney a small number of hyalinized glomeruli were found



Fig 11
Granuloma in the outer part of the cortex of a rat given 0 34 per cent Na_2PO_3F for sixteen weeks *H* and *E* stain × 210

In some cases with shrunken kidneys the dilatation of the tubules appeared to be less extensive Also, in animals that died spontaneously, only very slight dilatation was observed, although autolysis made accurate histologic analysis difficult

Finally, typical granulomas were found in many areas (Figs 8 and 9) They were always located in close relation to dilated Henle loops or dilated tubules, and after four weeks nearly all of them were seen in *the medullo-cortical zone*

Eighteen of the 32 rats given fluoride for 4 weeks had typical granulomas in the kidneys and two had granuloma like foci The granulomas consisted of rather large, round or slightly polygonal, reticular cells, and often one or two multinucleated cells in the section As a rule some lymphocytes and plasma cells were seen in the circumference (Fig 10)

The number of granulomas was increasing during the experiments and greatest in the groups in which animals received the highest dosage of fluoride Thirtytwo of the 43 rats surviving the fluoride dosage for 8–16 weeks showed granulomas After 12 and 16 weeks the granulomatous lesions occasionally occurred in the outer part of the cortex (Fig 11)

DISCUSSION

As appears *from the weight* data and the pathological examinations there were *no clear* differences as regards chronic toxic effect between *fluoride and monofluorophosphate* This is in striking contrast to the much lower acute toxicity of the latter ion, as referred to in the introduction

No pathological findings were made in the liver, spleen or adrenals while the *kidney* changes were of the same character and degree following both fluorides Previous experimental data have demonstrated that the liver is quite resistant to high doses of sodium fluoride (*Suvert & Phillips* 1959, *Shupe et al* 1960, and others), which, however, seemed to depend on the low fluoride concentration in the liver (*Wallace* 1953, *Zipkin et al* 1958, *Smith et al* 1960, and others) Our present data show that the *moderate* fluorine accumulation in the liver and spleen following the administration as PO_3F^- ions does not involve any toxic effects at the applied dosages

The similarity of the kidney changes following a high dosage of fluorine as NaF or Na_2PO_3F may indicate that the main part of fluorine passing the kidney after Na_2PO_3F administration is in the form of fluoride ions This is supported by the fact that *kidney* homogenate, as *well as homogenate of liver and small intestine*, have been found to split the PO_3F ion rapidly (*Ericsson*, current investigations)

While no occlusion of the nephrons could be demonstrated, the microscopic picture of the early lesion suggested a block close to the glomeruli, *i e*, in the transition zone between the ascending branch of the Henle loop and the tubulus contortus II Whether the juxtaglomerular apparatus played any rôle in the blockage of the tubule could not be determined, but morphologically the apparatus appeared more prominent than normally found by ordinary staining methods

The numerous cylinders found in the collecting tubules *of animals* given fluoride for long periods could possibly explain the more widely distributed dilatation seen in the late lesion The number *of inflammatory* cells was relatively moderate after four weeks and increased greatly later in the experiments The latter effect could have been the result of complicating infection This question cannot be answered, since we did not examine the tissue for growth of bacteria

The reason why the contracted kidneys contained few, if any, dilated tubules was probably related to cessation of function in the nephrons

The granulomas will be examined more closely and the results reported later

SUMMARY

Notable concentrations of radioactive fluorine were found in the liver and spleen of mice and rats following administration in the form of labelled monofluorophosphate ions (Na_2PO_3F), in contrast to the administration in the form of simple fluoride ions This raised the

question whether the PO_3F ion would exert special toxic effects on the liver or a chronic toxicity that was high in comparison with its acute toxicity

Male rats weighing about 100 grams were given *ad libitum* an adequate powdered diet with different fluoride content for periods up to 16 weeks In five groups the fluoride additives were, respectively 0 17 per cent Na_2PO_3F, 0 34 per cent Na_2PO_3F, 0 05 per cent NaF, 0 1 per cent NaF, no addition (control) 0 17 and 0 34 per cent Na_2PO_3F give the same fluorine contents as 0 05 and 0 1 per cent NaF, respectively (about 226 and 452 ppm F, respectively) The animals were given tap water containing about 0 1 ppm F *ad libitum*

The weight increase of the animals given the smaller fluoride additives was slightly impaired, that of the animals with the greater fluoride additives strongly impaired, without any consistent differences between groups receiving the same fluorine quantities in the form of Na_2PO_3F or NaF Two animals out of twentyfour in the 0 34 per cent Na_2PO_3F group and three animals out of sixteen in the 0 1 per cent NaF group died during the experiment

No pathological changes were found in any group on microscopic examination of the liver, spleen and adrenals In contrast, the kidneys were abnormal in most of the animals given fluorides, with the most severe changes associated with the highest doses and the longest survival periods In addition to the previously well known dilatation of the renal loops and ducts PAS positive casts were seen in pronounced cases in many dilated ducts and also typical granulomas in the medullo cortical zone and occasionally in the outer part of the cortex

In contrast to the comparatively low acute toxicity of sodium monofluorophosphate its chronic toxicity is thus of the same order and character as that of sodium fluoride

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THE SMALL LYMPH VESSELS OF THE LUNGS IN LYMPHANGIOSIS CARCINOMATOSA

By

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Received 21 v 65

During a previous investigation of the pulmonary lymphatics (3) it struck us that the ramifications of this system might be studied with

reported what we believed to be lymphatics in the alveolar septa, which are often described as being void of lymph vessels (8) We therefore thought it worth while to search for corroborating evidence in cases of lymphangiosis carcinomatosa —*Goldberg* reported the occurrence of lymphatics subintimally in veins in cases of lymphangiosis carcinomatosa of the spleen (4) as well as in portal veins of the liver (5) Whether the walls of blood vessels normally contain lymphatic pathways is a time honoured question (8) We were now also interested in finding out whether subintimal lymphatics occur in the pulmonary blood vessels for if they do, it would help to explain the changes in the pulmonary blood vessels in lymphangiosis carcinomatosa For instance, it is still debatable whether thrombosis of the blood vessels should be ascribed to a reaction to carcinomatous growth in the perivascular lymph vessels or to tumour fragments transported in the actual blood stream (9)

MATERIAL AND METHODS

The material consisted of 174 subjects with tumours that had metastasized to the lungs This series represented all cases of malignant tumours with pulmonary secondaries seen in 1962 at the department of pathology, Malmo general hospital (The town of Malmo has about 250 000 inhabitants and only one hospital which has a pathology department where 99 per cent of all persons dying in hospital are necropsied and 60 per cent of all who die in the town) In 1962 all together 1320 subjects were autopsied Of these 570 (43 per cent) had malignant tumours The frequencies with which various tumours metastasized to the lung are given in Table 1

In every case at least 5 sections of the lungs including at least one section of each lobe were available for histologic examination The sections were stained with haematoxylin erythrocin but in several cases of lymphangiosis carcinomatosa van Gieson and Weigert's stain for elastica or a combination of both stains were also used

In addition to these 174 cases with pulmonary metastases the material comprised

5 cases of myelogenous leucosis and 5 cases of lymphatic leucosis with pulmonary infiltration (not only from our 1962 material) as well as the lungs of 10 newborns (without tumours). In these cases, too, at least one section of each lobe of the lungs was available for histologic examination.

TABLE 1

Tumour	Number of cases	Cases with pulmonary metastases	Cases with lymphogenic spread of growth
Ca. coli et recti	69	19	8
Ca. ventriculi	60	15	8
Ca. bronchiale	56	28	16
Ca. mammae	49	23	20
Ca. viar. urin.	31	11	4
Ca. prostatae	27	9	8
Ca. uteri	25	5	3
Ca. renis	20	9	5
Ca. pancreatis	20	9	8
Ca. ovarii	18	5	1
Ca. hepatis	17	10	5
Ca. oesophagi	14	4	1
Malignant melanoma	6	4	2
Ca. thyreoidae	4	1	–
Reticulum cell sarcoma	6	5	
Lymphosarcoma	3	1	
Multiple myeloma	12	2	
Ca. vesicae felleae	8	3	2
Ca. laryngis	3	2	1
Ca. vulvae	2	2	2
Testicular tumour	2	2	–
Sarcoma of the chest wall	1	1	1
Osteogenic sarcoma	1	1	–
Ewing's sarcoma	1	1	1
Chondrosarcoma	1	1	–
Pleuramesothelioma	5	1	1

RESULTS

Of the 174 cases with pulmonary metastases, tumour growth was found in the lymph vessels in 97, in at least half of which the spread was predominantly lymphogenic. In none of these cases was tumour tissue found solely in the lymphatics, but invariably in association with tumour growth in the blood vessels and/or with large masses in the pulmonary parenchyma. Table 1 shows how often growth of the various types of tumour was seen in the lymphatics. It will be seen that most tumours can grow in the lymph stream of the lungs. Of the commonest tumours in the present material, cancers of the prostate, the pancreas, and the mammary glands showed a marked tendency to spread via the lymphatics in the lungs. In many cases the tumour spread simultaneously via the blood vessels and the lymphatics, but in most cases dissemination was either mainly lymphogenic or mainly haematogenic. In advanced lymphangiosis carcinomatosa thrombi were often seen in various parts of the blood stream. Sometimes the thrombi harboured

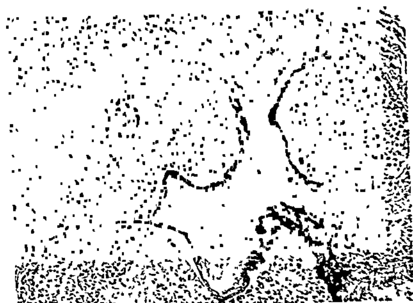


Fig 1
Malignant growth in periarterial lymphatics of lung in a case of hepatocellular cancer van Gieson × 75

tumour cells but only in a few cases was thrombosis of the blood vessels severe

In lymphangiosis carcinomatosa the course of the large lymphatic trunks (intrapleural, interlobular, perivascular and peribronchial) in the lungs can be readily recognized (Fig 1). Tumour masses in the large lymphatics were sometimes seen to extend out to the alveolar septa This infiltration was often continuous with the tumour within large lymphatics and gave the impression of fine branches of the latter But the tumour cells were often situated in endothelium-lined spaces not uncommonly wreathing blood capillaries in the alveolar septa (Figs 2 and 3) Large intraparenchymatous pulmonary metastases were sometimes seen to spread in the same way to the alveolar septa, while others were well outlined against the adjacent tissue —This type of spread was thus most often seen in endothelium-lined spaces and without loss of continuity with the tumour in the large lymphatics or with intraparenchymal metastases, but it was none the less often possible to trace the tumour far out into the alveolar septa On the other hand, the growth in large lymphatics was always more dominant than the spread in small vessels in the alveolar septa, and in several cases of lymphangiosis carcinomatosa no tumour growth at all could be demonstrated in the alveolar septa

In only one case was tumour demonstrable in a subintimal endothelium-lined space, and then in only one section (Fig 4) Unlike *Goldberg*

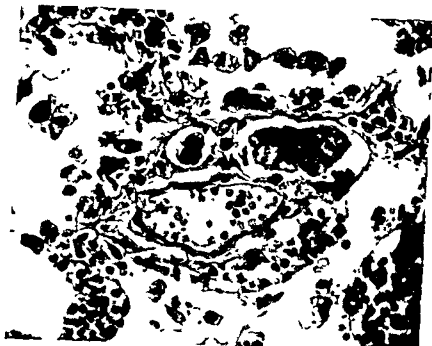


Fig 2

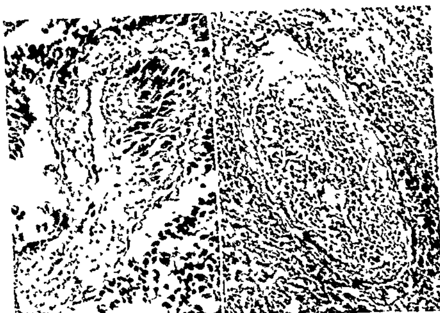
Cancer growth in vascular spaces around small blood capillary Same case as in Fig 1 van Gieson × 480

Fig 3 *Fig* 4

Figs 3 4

Fig 3 Cancer growth in endothelium lined vascular spaces along blood capillaries in alveolar septa Haem eryth × 1200

Fig 4 Cancer growth in small endothelium lined vascular space in wall of *vein* in a case of mammary cancer van Gieson × 115



Figs 5–6

Fig 5 van

Fig 6 ntima

(4), who demonstrated subintimal accumulations of lymphocytes and reticulocytes in the splenic veins, we were unable to trace such cells in any of the blood vessels of the lungs But tumours were often seen to invade the walls of blood vessels and to grow into their lumina This was often seen in cases in which the perivascular lymphatics were filled with masses of tumour, but then the tumour had, as a rule, also grown through the walls of the lymphatics invading the adjacent pulmonary parenchyma and the growth within the walls of the blood vessels was not limited to distinct vascular spaces It is true that the tumour had sometimes invaded the intima of the blood vessels but spared the media, but subintimal tumour buds of the type described by *Goldberg* in the splenic veins (Figs 5, 6) were never seen On the other hand, when the cancer grew in peribronchial lymphatics, tumour growth was often seen in small vascular spaces in the bronchial wall up to the submucosa

The series also included 6 cases of reticulum cell sarcoma, 1 case of lymphosarcoma and 1 case of multiple myeloma In the last 2 cases and in 3 reticulum cell sarcomas (in the remaining cases the pulmonary tumours appeared in the form of intraparenchymatous noduli) the infiltrates accompanied the lymphatics Cells from tumour masses around large lymph vessels often infiltrated the alveolar septa, but were never seen in the intima of the blood vessels (Figs 7, 8)

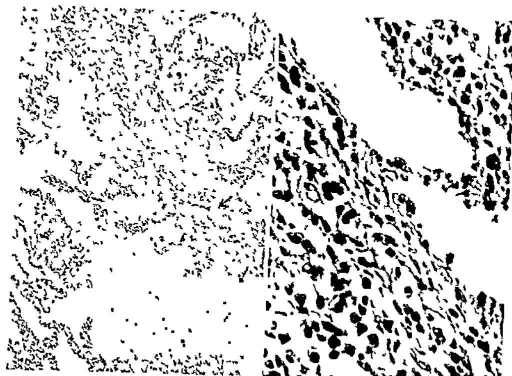


Fig 7 Fig 8

Figs 7 8

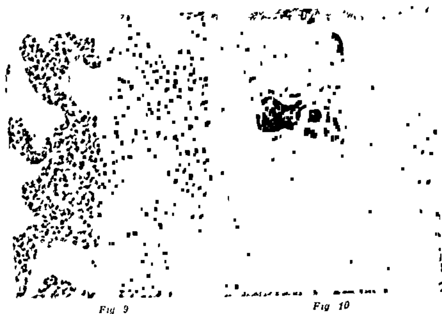
Fig 7 Infiltration of interlobular septa and alveolar septa in a case of reticulum cell sarcoma A few small lymphatics (indicated by the arrows) are seen in the interlobular septa Haem erythr × 85

Fig 8 Infiltration of multiple myeloma along lymphatic with valves Haem erythr × 480

Several of the findings made in malignant lymphoma were also made in cases of lymphatic leucosis Here, too, diffuse infiltration was seen along the lymphatic pathways and out into alveolar septa, but *not* subendothelially in the blood vessels Neither were the larger blood vessels plugged with lymphocytes In one case of lymphatic leucosis the clusters of lymphocytes normally occurring along the lympatics were markedly enlarged, but no other tumour infiltrate was seen *In* cases of myelogenous leucosis immature cells were seen particularly in the blood vessels, but in these cases, too, the alveolar septa were diffusely invaded and showed the same picture as in lymphatic leucosis (Fig 9) Otherwise lungs with predominantly haematogenous spread of tumour showed tumour cells in the blood capillaries here and there in the alveolar septa, but no diffuse infiltration of the septa by extension

The normally occurring groups of lymphocytes along the pulmonary lymphatics are increased in size in all sorts of irritation and occur, for example, subpleurally in scars Then lymphocytes are often seen to spread also out to the alveolar septa We often observed such a reaction within intraparenchymatous metastases in the lungs (Fig 10)

Coal dust is taken up by the pulmonary lymphatics and deposited



Figs 9 10

Fig 9 Leukamic infiltration of alveolar septa Myelogenous leucosis Haem erythr × 300

Fig 10 Lymphocyte infiltration of alveolar septa in pulmonary tissue with metastasizing squamous epithelial cancer van Gieson × 105

along them The deposits are often most dense in the clusters of lymphocytes along the lymphatic pathways Some of the lungs with metastases also showed severe anthracosis In such cases we saw distinctly how the coal dust accumulated along the lymphatic pathways and spread in alveolar septa near large lymphatics The pigment was often seen in the bronchial walls but never in the walls of the blood vessels, except for small amounts in the adventitia and outer part of the media

In newborns the pulmonary lymphatics are wide and readily recognized In none of the newborns in the present material could we demonstrate any *subintimal lymphatics in pulmonary blood* vessels, but we could not detect any lymphatics in the alveolar septa with certainty either

DISCUSSION

Among the most common types of tumours, it was particularly carcinoma of the prostate, pancreas and breast that showed a marked tendency to metastasize to the lungs via the lymphatics This is in accord with the previous report by workers in this field (*Spencer* (9), *Walther* (11) and others) On the other hand, we found only a few cases with advanced thrombosis and obstruction of blood vessels, changes considered by some authors to be almost obligatory in lymphangiosis carcinomatosa (*Hauser* (7))

This investigation gave support to the contention of an existence of lymphatics in the alveolar septa The growth of the tumour by extension in endothelium-lined spaces in the alveolar septa like branches from larger tumour-filled lymphatics suggested the existence of lymphatic pathways in the alveolar septa Considering how far they extended in the alveolar septa they can hardly be regarded as small lymph capillaries near large lymphatic pathways, but rather as a rich network of lymph capillaries within the alveolar septa The spread of malignant lymphomas and of lymphatic leucosis along the lymphatics and out to alveolar septa also argues for the occurrence of lymphatics in the alveolar septa In one case of lymphatic leucosis we saw only enlargement of the groups of lymphocytes normally occurring along the pulmonary lymphatics It is probable that in malignant lymphoma and lymphatic leucosis the malignant change starts in these very foci, whence it may presumably readily spread along the lymphatic pathways This sequence of events is seen in some inflammatory conditions of the lungs Thus, as a reaction to tumour growth in the lungs we saw enlargement of the accumulations of lymphocytes along the lymphatics, whence lymphocytes infiltrated the alveolar septa. In cases with haematogenous spread of cancer in the alveolar septa some of the capillaries contained tumour cells as well as red blood cells and did not show the more or less uninterrupted extensions of the tumour described above On the other hand red blood cells were not seen in the tumour-filled intraseptal "lymph capillaries" It is true that similar invasion of the septa was seen in myelogenous leucosis In cases of myelogenous leucosis the myelogenous, immature cells lie in the blood vessels and the picture with diffuse infiltration of the alveolar septa can easily be explained if it be borne in mind that it is the circulating blood that has become malignant (In our cases of lymphatic leucosis the larger blood vessels were not plugged with lymphocytes But lymphatic leucosis can produce the same picture as myelogenous leucosis by 'haematogenous spread" in the lungs) Finally, the distribution of the coal dust in the pulmonary parenchyma also supports the assumption of lymph capillaries in the alveolar septa The coal dust was situated like infiltrates of malignant lymphoma along lymphatics, particularly in accumulations of lymphocytes, from which they spread out to the alveolar septa

The investigation provided no evidence of subintimal lymphatics in the veins or arteries In the only case in which we saw a cancer-filled capillary in the intima of a vein, it might as well have been a blood capillary as a lymph capillary If it was a lymph capillary it might have been one draining into a blood vessel Such connections have often been described, but they have not been generally accepted (8) Lymph vessels are readily demonstrable in the walls of the bronchi up to the submucosa, so that if lymphatics really exist in the walls of blood vessels, it should have been possible to detect them there The fact that we were not able to demonstrate infiltrates of malignant lymphoma or lympha-

lic leucosis in the intima of the blood vessels also argues against the existence of lymph capillaries subintimally In no instance did we see cancer filled lymphatics bulging into blood vessels, a finding made in the spleen by *Goldberg* and by one of us (1) But the wall of the veins in the spleen has no muscular wall and consist of a single layer of endothelium cells (2)

Lymphangiosis carcinomatosa was described as early as 1829 by *Andral* and it was *Troisier* (1873) who gave it its name (9) Since then much has been written about this type of dissemination of tumours, particularly with regard to the lungs Various theories have been put forward to explain the simultaneous haematogenous spread and the widespread thrombosis and occlusion of the blood vessels It would have been tempting to ascribe the changes in the blood vessels to tumour growth in the subintimal lymphatics, but no evidence for such an assumption could be produced

SUMMARY

A large autopsy series (174 cases) of lungs with tumour metastases, particularly cases of lymphangiosis carcinomatosa, was studied histologically for lymphatic vessels in the alveolar septa and subintimally in the blood vessels The investigation gave support to the contention of an existence of lymph capillaries in the alveolar septa but not of an existence of lymph capillaries subintimally in the blood vessels

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ON THE VIABILITY OF TUMOUR CELLS IN ARTIFICIALLY PRODUCED SUSPENSIONS

By

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Received 9 vi 65

The degree of dispersion and the viability of cells in artificially prepared suspensions are factors of great interest in experimental cytology and oncology Especially when studying tumour spread by metastasis these factors are of profound importance They are, however, often not critically considered

Cell suspensions from solid tissues may be produced either by mechanical treatment of the tissue (*Schneider & Potter* 1943, *Schrek* 1944 *Palade & Claude* 1949 *Bucher et al* 1951) or by chemical or enzymatic breaking down of the intercellular substances (*Medawar* 1941,
1957 *Rinaldini* 1958, 1959, *Bo*
drastic maltreatment of the t
representative of the tissue, a
numbers

Usually mechanical suspensions have few viable cells and show great variations in degree of dispersion (*Anderson* 1953, *Laws & Stickland* 1956) On the other hand *Schrek* (1944) found a high percentage of viable cells in mechanical suspensions from thymic and lymphoid tissues The structure of the original tissue may influence the quality of these suspensions (*Kaltenbach & Kaltenbach* 1956)

Enzymatically produced suspensions from several neoplastic tissues prepared with trypsin and DNase according to *Madden & Burk* (1961) show a high cellular viability and a high degree of dispersion However trypsin is noxious to cells (*Weiss* 1958 *Levine* 1960 *Lucy* 1960 *Vaitkevicius et al* 1961) It cannot be excluded that cells are damaged without changes in their stainability with vital dyes

Most studies on the viability of cells in suspensions are based on results with vital staining However it was shown by *King et al* (1959) on Ehrlich tumour cells that the vital dye Trypan Blue recorded only late stages of cell injury earlier stages such as loss of capacity for division or respiration were not revealed

In our laboratory suspensions from transplantable tumours are used in different experiments, including studies on tumour spread by metastasis We found it important to supplement vital staining procedures with a better defined parameter for cellular viability, *viz* the incorporation of labelled precursors in nucleic acid metabolism With *in vitro* incubation, the nucleic acid metabolism of tumours cells *in suspension* was correlated to that of the tumour *in vivo*, and then the technique was used in experiments comparing degree of dispersion and viability

in enzymatically and mechanically produced suspensions from different tissues

MATERIAL AND METHODS

rhabdomyosarcoma,

a Fischer

[illegible]

(/mM) and adenine 2 8-³H (The Radiochemical Centre,

er (1945) was

measured with

[illegible]

the emulsion, sections with undiluted Giemsa and sedimented cells with one per cent aqueous Toluidine Blue

Label index (L I) The percentage of labelled cells was determined by counts in

[illegible]

hy, and the remainder was used for

the suspension was used for deter-

was sedimented onto slides for auto radiography

[illegible]

per cent Parker 199 with antibiotics, and 0 1–0 2 ml of isotope solution The tubes

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ON THE VIABILITY OF TUMOUR CELLS IN ARTIFICIALLY PRODUCED SUSPENSIONS

By

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PER M LUNDIN and KLAS NORRBY

Received 9 vi 65

The degree of dispersion and the viability of cells in artificially prepared suspensions are factors of great interest in experimental cytology and oncology Especially when studying tumour spread by metastasis these factors are of profound importance They are, however often not critically considered

Cell suspensions from solid tu
ment of the tissue (*Schneider &*
Bucher et al 1951) or by chemic
substances (*Medawar* 1941 *Mos*
1957 *Rinaldini* 1958 1959 *Boyse*
drastic maltreatment of the tissu
representative of the tissue and
numbers

Usually mechanical suspensions have few viable cells and show great variations in degree of dispersion (*Anderson* 1953 *Laws & Stickland* 1956) On the other hand

staining However it was shown by *King et al* (1959) on Ehrlich tumour cells that the vital dye Trypan Blue recorded only late stages of cell injury earlier stages such as loss of capacity for division or respiration were not revealed

In our laboratory suspensions from transplantable tumours are used in different experiments, including studies on tumour spread by metastasis We found it important to supplement vital staining procedures with a better defined parameter for cellular viability *viz* the incorporation of labelled precursors in nucleic acid metabolism With *in vitro* incubation the nucleic acid metabolism of tumours cells in suspension was correlated to that of the tumour *in vivo* and then the technique was used in experiments comparing degree of dispersion and viability

almost 100 per cent single cells, with only isolated clusters of 3–4 cells M suspensions from the mouse sarcoma showed many large clusters, those from the pituitary tumour rather few, but the thymic M suspensions approached 100 per cent single cells

In Papanicolau stained smears, E- and M suspensions from the pituitary tumour and from thymus both showed well preserved cell structures but with the mouse sarcoma, cells were well preserved in the E suspension only

In Table 2 is presented the viability of cells, as found by vital dye reactions in E and M suspensions from the same samples of the three *different tissues*

TABLE 2

Viability of Cells in Enzymatic and Mechanical Suspensions from the Same Tissue Samples Vital Staining before and after Incubation at 37° C in vitro

Tissue	Enzymatic				Mechanical			
	before		after		before		after	
	TB	NR	TB	NR	TB	NR	TB	NR
Mouse sarcoma	91	79	87	85	11	8	5	4
Pituitary tumor	93		91		34		38	
Thymus	97		80		99		89	

TB = Trypan Blue NR = Neutral Red Viability index in per cent

All E-suspensions had a V I above 90 per cent initially with little *change* after incubation for one hour M suspensions from mouse *sarcoma* and rat pituitary tumour both gave a very low V I but the M suspension from thymus had as high a V I as the corresponding F-suspension

Thus we find the E-technique superior for the two tumours studied but with *thymic tissue the* M technique gives just as good results The solid epithelial pituitary *tumour has a better V I* than the mesenchymal sarcoma when the M technique is used Earlier observations that the structure *of the* original tissue may influence the outcome of an M suspension *are confirmed* The more pronounced the cellular cohesion the worse the results to be expected using M suspensions

The difference between E- and M suspensions is also evident when the incorporation of thymidine in mouse sarcoma cells is studied *in vitro* (Table 3) The E-suspension gives a L I above that of the M suspension at every radioactive concentration tested

Also in vitro L I increases with increasing dose of isotope but the increase is not regular However individual tumours may have growth fractions of different size, and the number of cells incubated is not the same in the different experiments

Using the E-technique L I here shows that mouse sarcoma cells incubated in vitro are capable of synthesizing DNA in a percentage

were gassed with CO_2 to pH 7, stoppered and placed in a rack, rotating at 0 6 rpm Incubation took place in the *dark* at 37° C for one or three hours Thereafter V I was determined Cells were then washed twice with Parker 199 and sedimented onto slides *for autoradiography*

RESULTS AND DISCUSSION

After labelling with thymidine or adenine *in vivo* the mouse sarcoma showed numerous labelled cells especially at the periphery Twentyfour hours after injection of adenine many cells presented silver grains over nuclei only, but others clearly showed a cytoplasmic label

Results from the *in vivo* experiments with the mouse sarcoma are given in Table 1 It is seen that L I increases with increasing dose of thymidine This is more pronounced after thymidine of high specific activity, when less inactive thymidine is administered The higher L I after 24 hours may be explained by a small growth fraction for the tumour (*Mendelsohn* 1962)

TABLE 1

Incorporation of Tritiated Thymidine into Mouse Sarcoma in vivo *Specific Activity of DNA and Label Index for Solid Tumour and for enzymatic Suspension from the Same Tumour Viability and Label Indices in per Cent*

	Dose of isotope µC/g body weight	Tumour tissue: Specific activity cpm/mg DNA	Tumour tissue: Label index	Cell suspension: Viability FB	Cell suspension: Viability index NR	Cell suspension: Specific activity cpm/mg DNA	Cell suspension: Label index
	1	2112	19	92		2786	18
I	2	3344	26	92		3204	22
	10	4992	36	89	87	3000	31
	1	1260	9	93	90	2520	7
II	3	4470	17	91	85	8510	20
	10	10730	19	94	80		18

In experiment I thymidine with a specific activity of 0 36 C/mM was injected and the animals were killed after 24 hours In experiment II the specific activity of thymidine was 6 C/mM and the animals were killed after 1 hour

When comparing L I and specific activity of DNA for solid tumours and corresponding E-suspensions, the values are essentially of the same magnitude throughout, except for a somewhat higher specific activity in some of the suspensions The results were quite similar with adenine Thus we conclude that when an E-suspension of this type is produced there is no important relative loss of cells with capacity of proliferation, with one exception Few meta- and anaphases were seen among the centrifuged cells Many cells in mitosis thus are lost during the procedure

In the *in vitro* experiments E- and M-suspensions from the two tumours and from normal thymus were compared

Concerning the degree of dispersion, all E-suspensions consisted of

SUMMARY AND CONCLUSIONS

Cell suspensions have been produced from three different tissues, *viz* a mouse sarcoma, a rat pituitary tumour and normal rat thymus The viability of cells in enzymatic (E-) and mechanical (M-) suspensions produced from the same tissue samples was compared Cellular viability was studied with vital dye reactions, using Trypan Blue and Neutral Red, and for the mouse sarcoma also with autoradiographic determinations of the incorporation of tritiated thymidine or adenine *in vitro* For comparison the incorporation of labelled precursors was also investigated on the sarcoma *in vivo*, using autoradiography and radiochemical determinations of the specific activity of DNA

The enzymatic suspensions from the two tumours were markedly superior to the mechanical suspensions in degree of dispersion and in morphological preservation of cells The enzymatic suspensions had about 90 per cent viable cells against some 10 30 for the mechanical suspensions, as measured with vital staining procedures Enzymatic suspensions from the mouse sarcoma showed an incorporation of thymidine or adenine *in vitro* of the same magnitude as the tumour *in vivo*

It is concluded that the determination of a label index after *in vitro* incorporation of thymidine records differences in cellular viability not found with vital dyes Further, such determinations are directly related to the ability for division of single tumour cells, which is the main problem when suspended tumour cells are used experimentally

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well comparable to the tumour *in vivo*. Judging from a cytoplasmic label in some cells after incubation with adenine, a certain RNA synthesis also occurs.

All suspensions presented in Table 3 have V.I.'s of the same magnitude before and after incubation, yet L.I. varies when dose of isotope and incubation time are identical. In later experiments (to be published) it has been shown that slight variations in incubation conditions may change the L.I. considerably while V.I. remains unchanged, in samples from the same suspensions. Thus L.I. records another level of viability where difference are not shown with V.I. It is not necessary, however, that cells are damaged irreparably when L.I. is low. Under certain conditions metabolic activities may be only temporarily suppressed.

TABLE 3

Viability of Cells in Enzymatic and Mechanical Suspensions from the Same Sample of Mouse Sarcoma. Incorporation of Tritiated Thymidine at 37° in vitro. Label Index in per Cent

Dose of isotope µC/ml	Incubation time hours	Number of cells mill/ml	Enzymatic Label index	Mechanical Label index
0.1	1	0.2	10	3
1.0	1	0.1	10	3
1.0	1	7	27	
1.0	3	7	32	
2.0	1	0.2	22	5

When in experimental oncology the viability of single tumour cells is studied the final interest of course concerns the ability for division. Preliminary results show that cells in an E-suspension from the mouse sarcoma have a very high capacity to multiply *in vivo*. Thus when diluted samples, statistically expected to contain 400, 30 and 3 cells, were injected subcutaneously into normal mice, tumours appeared in 100, 60 and 20 per cent, respectively (*Norrby*, to be published). This is a high number of takes as compared to results with another isologous system used by *Madden & Burk* (1961). They had no takes with less than 100 cells.

Host factors, however, certainly can prevent or delay the multiplication of cells which otherwise must be considered viable. For this and other reasons it is very difficult to test *in vivo* the capacity of single cells to divide, and we must to a large extent rely on *in vitro* procedures. Although we cannot assume that the synthesis of DNA always destines a cell to divide the study of such a synthesis is certainly the best method available with relation to cell division. Further studies on the DNA-synthesis of tumour cells during the production and storage of suspensions are under way.

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MACROPHAGE RESPONSE TO EPIDERMAL ABRASION IN SARCOIDOSIS

Its Relation to the Patient's Kveim Reactivity

By

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Received 30 vi 65

The mononuclear phagocytes comprise a variety of cell types ranging from monocytes of the blood to histiocytes and epithelioid cells Monocytosis is often, but not regularly, encountered in sarcoidosis as well as in other proliferative disorders of the reticuloendothelial system (*Gravesen* 1942, *Putkonen* 1943) On escaping from the vessels, the monocytes are transformed into macrophages The latter are indistinguishable from histiocytes (*Carrel & Ebeling* 1926) After engulfing foreign agents that resist digestion, macrophages are transformed into epithelioid cells and giant cells (*Cohn & Benson* 1964). This is how sarcoid granulomas and also Kveim reaction papules are formed

Histiocytes, the tissue macrophages, have not been studied so intensively as the other types of mononuclear phagocytes in sarcoidosis In 1951, *Rebuck*, the inventor of the skin window technique for the study of cell dynamics in inflammation made the interesting observation that in a case of sarcoidosis the skin window coverslip at the six-hour stage of inflammation was made up "almost exclusively of masses of histiocytes (*Rebuck, Smith Jr & Margulis* 1951) In recent studies of 102 sarcoidosis patients *Mlczoch & Kohout* have emphasized that the hyperactive "lymphohistiocytic" reaction is a characteristic of sarcoidosis and has potential diagnostic value The percentage of these large mononuclear cells tended to be higher in the untreated acute cases than in the chronic cases or in patients treated with corticosteroids (*Mlczoch & Kohout* 1962, 1964)

Mlczoch & Kohout did not test their patients with Kveim antigen The development of a palpable Kveim reaction might, however, be more directly correlated with the mononuclear response on the skin window coverslips than the clinical estimation of the activity of sarcoidosis

This is a part of a sarcoidosis project directed by *T Putkonen* M D., M P H., and sponsored by the *Finnish Medical Research Council*

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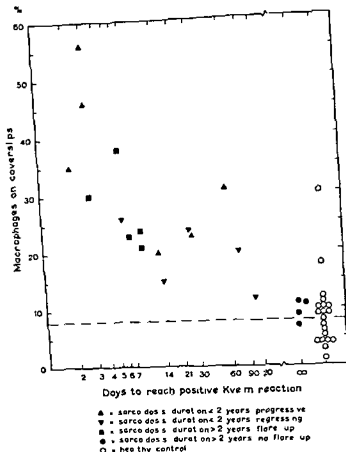


Fig 1

cent saline
patients in
large mono

RESULTS

At the six hour-stage of inflammation the skin window coverslip contained predominantly polymorphonuclear granulocytes and large mononuclear cells both in the sarcoidosis groups and in the control group of healthy persons The sarcoidosis patients, however, showed markedly more large mononuclears than did the controls, the mean of the sarcoidosis patients and that of the controls were 24 and 8 per cent.

The possibility of timed Kveim testing in our hospital made a comparison between the mononuclear response, Kveim reactivity and clinical condition of the sarcoidosis patient feasible

MATERIAL AND METHOD

The series consisted of 20 cases of sarcoidosis and an equal number of healthy volunteers matched by sex and age The age range was from 7 to 70 years with a

two years duration The second group consisted of 9 patients suffering from chronic *sarcoidosis*

These groups were further subdivided into two subgroups according to the activity of the disease

Group 1 a This subgroup consisted of five women and one man suffering from early *progressive* sarcoidosis All six patients had enlarging hilar lymph nodes Four had active erythema nodosum and two had according to their recent case histories, had erythema nodosum Sarcoid scars had appeared in two patients The only man in this group had neurological sarcoidosis, a progressive spinal paralysis In five of the patients biopsies could be obtained all showing sarcoid granulomas

Group 1 b This subgroup comprised five women with *regressing* sarcoidosis of less than two year s duration They all showed regressive roentgenologic changes compatible with pulmonary and/or hilar sarcoidosis In two of them mediastinal lymph glands were biopsied—both glands displayed sarcoid granulomas According to their past history one of the patients had had sarcoid scars three erythema nodosum and three ocular sarcoidosis

Group 2 a This subgroup consisted of one woman and four men with chronic sarcoidosis showing a *flare up* of their disease at the time of the skin window examination All had an active pulmonary and/or hilar sarcoidosis two had lupus pernio and in one sarcoid scars were developing In addition one man had an expansive intracranial process probable of sarcoid nature Four of the patients also had histological evidence of sarcoidosis

Group 2 *b* The last subgroup of four women had a rather stationary chronic sarcoidosis with advanced pulmonary disease showing regressing hilar lymphadenitis In every case histological evidence of sarcoidosis was obtained One of the patients with chronic lupus pernio had according to her case history had erythema nodosum

All the subacute sarcoidosis patients were Kveim positive *i e* showed a characteristic skin reaction to Kveim antigen Of the patients suffering from chronic sarcoidosis only those having a flare up were Kveim positive whereas all four patients in Group 2 b were Kveim negative Kveim antigen No 110 was used in the testing of all the patients This antige
patient with chronic lymphonodal sarc
pared with the value of 40 of the Type I

The standard test dose was 0 1 ml of antigen diluted 1 10 by wet weight of tissue Tests were then followed until the reactio
A sarcoid tissue reaction was always fou
mature Kveim papule At the time when the
were also subjected to quantitative Mantoux tuberculin tests using up to 100 T U of OT and PPD

Within three months of the injection of the Kveim antigen the skin window examination differential count of blood leucocytes and paper electrophoretic analysis of serum proteins were performed

There was a slight trend of hyperproteinaemia in the more chronic cases The median of total serum proteins in Groups 1 a 1 b 2 a and 2 b were 7 2 7 5 7 5 and 7 8 g per cent respectively In the albumin globulin ratio and in the gamma globulin level there was virtually no difference between the groups

For the skin window examination the forearm was washed with ethanol A standard epidermal lesion 5 mm in diameter was made with a finishing dental burr (*Mustakallio & Reechardt* 1964) The lesion was abraded to a depth where the capillary loops of dermal papillae became visible without oozing of blood A sterile coverslip was applied directly on the moist lesion and left for six hours fixed to the

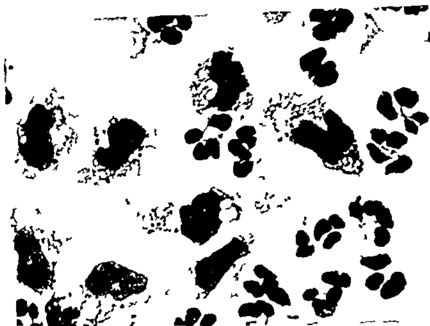


Fig 3.
Appearance of a skin window two weeks after injection of Alcian blue. Only the vacuolated macrophages have ingested the dye in the form of granules of varying size × 1200

median monocyte count in Group 1 a was 9 in Group 1 b 7 in Group 2 a 9.5 and in Group 2 b 5 5 per cent The correlation between the skin window mononuclears and the blood monocytes is illustrated in Fig 2 The most fulminating sarcoidosis patients of this series having 56 and 46 per cent of large mononuclears on the coverslips had blood count of monocytes and total leucocytes within normal limits

There was no correlation between the mononuclear response on the skin window and the tuberculin reactivity of individual patients Neither the total proteins the albumin globulin ratio nor the gamma globulin level showed a correlation with the mononuclear response at the individual level

The large mononuclear cells appeared to be phagocytes In persons with dark complexion they often contained melanin granules In addition the experiments with Alcian blue revealed that two weeks after injection only the large mononuclears contained the dye (Fig 3) For this reason they can be called macrophages

DISCUSSION

The macrophages appearing on the skin window coverslips during the initial six hours of inflammation are either histiocytes (*Becker Kudo Argenlo & Fischer* 1961) or transformed monocytes (*Paz &*

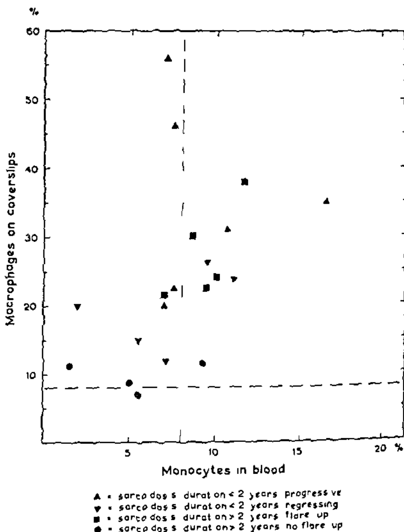


Fig 2

respectively Only occasional, small lymphocytes and eosinophils were seen on the coverslips at the six-hour-stage both in the sarcoidosis patients and in the controls

The large mononuclear cells on the coverslips showed a significant negative correlation with the time required for Kveim papules to reach the diameter of 5 mm (Fig 1) In progressive early sarcoidosis (Group 1 a) the median of the mononuclear count was 33 per cent, in regressing subacute sarcoidosis (Group 1 b) 20 per cent, in chronic sarcoidosis with a flare-up (Group 2 a) 24 per cent and in chronic stationary sarcoidosis (Group 2 b) 11 per cent

In patients with an equal Kveim reaction time, the more acute cases had higher counts of the large mononuclear cells

Blood monocytosis exceeding 8 per cent was found in 10 out of the 20 sarcoidosis patients It was independent of the total leucocyte count, which varied between 3800 and 8600 cells per ml (mean 5600) *The*

SUMMARY

The macrophage response to epidermal abrasion was studied by the skin window technique in 20 Kveim tested sarcoidosis patients and in an equal number of healthy controls

[illegible] hours of inflammation induced by epidermal

A significant correlation was found between the relative macrophage count and the rapidity with which the Kveim papule attained the diameter of 5 mm The dependence of macrophages from the level of blood monocytes was less striking

Intradermal injection of Alcian blue two weeks before the skin window examination afforded evidence for the histiocytic nature of a part of the emigrated macrophages

The hyperactive macrophage response to epidermal abrasion in sarcoidosis does not have the diagnostic specificity of the Kveim test It reflects the activity of the disease

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Spector 1962) or belong to both cell types The six-hour-interval was chosen in order to avoid a potential change of lymphocytes to macrophages, a transformation claimed to begin 8 hours after the trauma (*Rebuck, Smith Jr. & Margulis* 1951, *Braunsteiner, Paerlan & Thumb* 1958). At the six-hour-stage only occasional small lymphocytes were found, in conformity with the observations of *Leder & Nicolas* (1963) The small lymphocytes showed no evidence of transformation to phagocyte or blast forms.

The controlled abrasion of the epidermis with a dental burr was used because it avoids direct contamination with blood cells oozing from capillaries severed with a knife E g blood-borne lymphocytes and erythrocytes are seen in Fig 5 of *Mlczoch & Kohout* (1962)

The experiments with Alcian blue suggest that a part of the macrophages on the coverslips are in fact dermal histiocytes, Alcian blue is known to be engulfed within a few days by dermal connective tissue cells (*Bailie & McManus* 1963)

The macrophage response to the nonspecific abrasion trauma indicates an increase in the phagocytic capacity of skin in sarcoidosis In conformity with the results obtained by *Mlczoch & Kohout* this was found to occur in patients suffering from active sarcoidosis, whereas in chronic stationary cases the macrophage response was weaker and approached the level of healthy subjects

Monocytosis has also been reported to occur more often in "active" than in "stationary" cases of sarcoidosis (*Gravesen* 1942) In our series the patients suffering from sarcoidosis of more than two years' duration showed a rather good correlation between monocytes and tissue macrophages The chronic cases with a flare-up tended to have higher blood monocyte counts than the more stationary ones In acute sarcoidosis a flux of monocytes from the blood toward the tissues may predominate A follow-up of blood monocytes and skin macrophages before and after the Kveim test might be more rewarding than a single count (*Putkonen, Jokinen & Mustakallio* 1965)

The macrophage response corresponded to the timed Kveim reaction even better than the blood monocytes Apparently the greater the initial number of skin macrophages induced to proliferate by the Kveim antigen, the sooner the reaction papule will reach the diameter of 5 mm

The hyperactive macrophage response to epidermal abrasion is not specific for sarcoidosis It can be seen in some other proliferative disorders of the reticuloendothelial system and even in apparently healthy persons Moreover, in chronic stationary sarcoidosis with a negative Kveim test the macrophage response is not exaggerated

In sarcoidosis the macrophage response to epidermal abrasion does not have the diagnostic value of the Kveim test, but indicates the activity of the sarcoidosis quite well

MATERIAL AND METHODS

, and were col-

per ml 100
diluted with
this P H A
were tried,
mitogenic
of this P H A was 0 4 ml per 10 ml of undiluted plasma The cultures were incubated at 37° C for 64 hours, and tetracycline (Tetracyn, Pfizer) was later added to the cultures for 4 hours The concentration of tetracycline in the cultures was varied between 0 5–1 35 mM Subsequently the culture was shaken and washed twice in physiological saline centrifugated at 800 r p m for 5 minutes, the pellet produced

orcein method for bright field examination It should be noted that the use of neutral fixative as above is important for the preservation of tetracycline fluorescence, 95 per cent ethanol or a mixture of ethanol and aether (1 1) can also be used,

finally fixed and stained cells

cases hypotonic treatment was added according to method 2 and in the remaining 10 cases the cultures were treated with Colcemid From these slides ordinary chromosome analysis with karyotyping was carried out

RESULTS

All control cultures were seen to have a good growth and typical well-formed P H A blast cells In those samples from ten individuals which

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INCORPORATION OF TETRACYCLINE IN PHYTOHAEMAGGLUTININ BLAST CELLS

By

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Received 15 vii 65

When human peripheral lymphocytes are cultured *in vitro* under the influence of the bean extract phytohaemagglutinin (P H A), the small lymphocyte can undergo transformation into a large basophilic, blast-like cell capable of cell division (*Nowell* 1960, *McKinney et al* 1962, *Elves & Wilkinson* 1963) The morphological characteristics of this blast cell are the following the nucleus is loosely structured and has a great nucleolus, the cytoplasm is markedly basophilic and shows coarse sudanophilic granules, the cell shows a great resemblance to myeloblasts or the cells referred to as reticulum cells when seen in blood marrow aspirates The P H A blast cell is very similar to the abnormal lymphoid cells seen in the peripheral blood of patients with glandular fever and other infectious conditions (*Elves et al* 1964, *Gropp & Fischer* 1964) A special feature of the P H A cell is that it has an increased number of mitochondria and these are of giant size as is shown by electron microscope studies (*Elves et al* 1964, *Olsson* 1964) *Hirschhorn et al* (1965) reported that the P H A cells contain also a rich amount of granules which could be stained with a method for acid phosphatase activity They are called the lysosyme-like structures

When tetracycline is administrated to the living organism it can be seen to deposit in necrobiotic changes of different origin (*Hakkinen & Hartiala* 1959, *Vassar et al* 1960, *Mustakallio* 1962, *Saxen & Vainio* 1964) and in normal, growing bone connective tissue (*Harris et al* 1962) and in pathological calcifications (*Hakkinen & Lindgren* 1963) At intracellular level tetracycline has been seen in the mitochondria of cultured renal cells of monkeys (*DuBuy & Showarre* 1961)

The present paper is a report of the study on tetracycline *in vitro* incorporation in leucocyte culture in which the cells are under the influence of phytohaemagglutinin and the blast cells with great amounts of cytoplasmic organelles are developed

This work was supported by grants from the *Finnish Cancer Association* and *Sigrid Jusélius Stiftelse*









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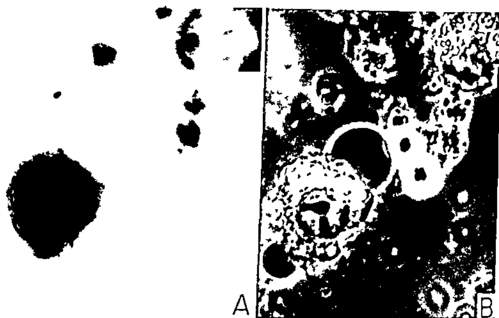


Fig 1

Living phytohaemagglutinin blast cells incubated for four hours with tetracycline in culture medium A Fluorescent light Several small, circularly arranged, bright fluorescent particles Weakly fluorescent nuclear shadows close to the bright areas B Phase contrast view of the same field The bright particles located in cytoplasm Magnification 2200 ×

were treated with the complete method of chromosome analysis, the normal human karyotype 46/XX or XY was observed

The direct observation with fluorescent illumination showed that the cultures which were incubated with tetracycline contained, under the microscope, very many yellow fluorescent areas These were composed of all small round particles which were arranged in ring form In the middle of these were large ball like corpusles which were seen to have a distinct boundary (Fig 1 A) When the illumination was changed to ordinary light and phase contrast optics on the same field as had been examined by fluorescence microscopy was used for the examination it could be seen that the large, weak, fluorescent balls were nuclei and the heavy fluorescent particles were located in the cytoplasm forming a ring around the nucleus These particles showed a negative phase contrast effect (Fig 1 B)

When the cultures were fixed and spread on the slides the same ob-

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Fig 2

Fixed phytohaemagglutinin blast cells The cells have been incubated for 4 hours with tetracycline and treated for 20 minutes with hypotonic sodium citrate solution before fixation A Fluorescent light Group of small bright fluorescent particles on dark background B Same field Phase contrast view In addition to the fluorescent points several nuclear shadows of different shapes are seen C Same field Acetic orcein stain Nuclei are stained dark the fluorescent area not at all Magnification 2200 × in all figures




A
B


C

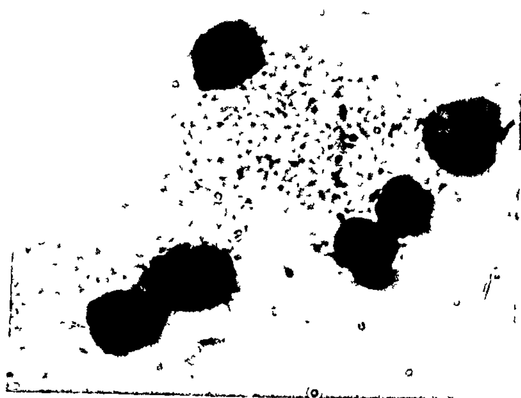


Fig 3
Phytohaemagglutinin blast cells Method for succinate dehydrogenase with nitro BT Many mitochondrias present Magnification 2200 ×

servations could be made and finally confirmed when the preparations were stained with acetic orcein and studied with ordinary bright field illumination

When the above procedure was extended by hypotonic treatment with 0 95 per cent trinatriumcitrate and examined in an identical way the following observations were made There were as many bright yellow fluorescent areas as in the living culture However, these areas were not seen to form ring-like groups, but were concentrated to form a round area in which there were small pin-shaped particles under approximately 0 5 μ in size, which were randomly dispersed (Fig 2A) Besides this bright fluorescent areas there were many weak fluorescent homogenous areas which, when studied by phase contrast, were seen to be nuclei The bright fluorescent areas showed a strong contrast in the phase picture (Fig 2B) The sizes of these areas were approximately the same as the sizes of the nuclei Finally when the preparations were stained with acetic orcein and the same areas were examined on an ordinary bright field it could be stated that the weak fluorescent areas were nuclei, but the bright fluorescent areas were not stained at all (Fig 2C)

The succinate dehydrogenase method showed a strong reaction in the cytoplasm of the P H A cells and indicates the presence of a great amount of mitochondrias (Fig 3) The Gomori method showed also an amount of acid phosphatase containing granules When these organelles were compared with the particles containing tetracycline, it could be seen that the mitochondrias had the same size and form as the particles presenting tetracycline fluorescence This could be ascertained also with dark field illumination observations in unstained slides The lysosome like bodies were smaller and less numerous than the tetracycline containing corpusles

The cultures treated with Colcemid were seen to contain the same bright and weak fluorescent areas, but no fluorescence could be seen in the chromosome The spreading of chromosomes when the methanol-ethanol acetone fixing solution was used was not satisfactory for karyotyping

DISCUSSION

The results show that tetracycline is deposited in cytoplasm and can be moved from the cells by hypotonic treatment The size and morphology of the particles in which tetracycline is deposited are similar to the giant mitochondria which are seen by electron microscopy in P H A cells (*Elves et al* 1964, *Olsson* 1964) Furthermore it should be pointed out that no tetracycline at all is seen in the nuclear material, nor in interphase nuclei or in chromosomes Once it was hoped that the tetracycline fluorescence might be an indicator in neoplasm detection, but the only basic requirement of this being the specific nuclear depositing of the drug no experimental basis has been demonstrated by this and other studies (*Vassar et al* 1960)

Tetracycline can suppress the growth of lymphocytes *in vitro* when administered in concentrations comparable to those used for therapeutic purposes in Man (*Lindgren* 1964) As the drug is deposited in the mitochondria this growth inhibition may be due rather to the prevention of mitochondrial functions than to direct mitosis inhibition

It has been shown that P H A cells are capable of producing gamma globulin (*Elves et al* 1963) and as the development of cells showing the same morphological characteristics as the P H A cells is seen *in vitro* under the influence of various antigens, *e g* tuberculin (*Permain et al 1963*) tetanus toxoid diphtheria toxoid, smallpox vaccine (*Elves et al* 1963) and staphylococcal toxins (*Ling & Husband* 1964), the growth of these cells can be considered to be caused by immunological mechanism (*Ling & Husband 1964*) Thus such giant cells as are seen in glandular fever and other infectious conditions *in vivo* may have a certain important role in inflammatory response by producing antibodies The prevention of this function may have caused by tetracycline administration



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SUMMARY

When tetracycline was added to short-term cultures of human peripheral leucocytes, the giant blast cells which are grown under the influence of bean extract phytohaemagglutinin were seen to collect the drug in their cytoplasm Due to its fluorescent property tetracycline could be detected easily in the cytoplasm in particle form These particles could be separated from the nuclei by hypotonic treatment, and were formed to have the same size and form as mitochondrias Granules staining for acid phosphatase activity (lysosyme-like) could be detected in the cytoplasm of blast cells No tetracycline incorporation could be seen in these granules, nor in interphase nuclei or in metaphase chromosomes

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THE CALCIFICATIONS OF THE HUMAN THYROID GLAND

A Histologic and Biophysical Study

By

ILMARI LINDGREN and CURT LAGERGREN

Received 15 vii 65

In a study of the literature it is soon discovered that conceptions of the occurrence and nature of thyroid calcifications are highly divergent *Wegelin* (1926) was of the opinion that thyroid calcification was an uncommon occurrence, while *Fassbender* (1956) for instance, found thyroid calcifications to be relatively frequent especially in elderly people Certain scientists have considered thyroid calcifications to bear some relation to thyroid cancer (*Batsakis et al* 1960) and they have maintained even that roentgenologically visible calcifications are of diagnostic value in cases of thyroid cancer (*Holtz & Powers* 1958, *Gerard Marchant et al* 1962) while others (*Wegelin* 1926) have assumed that calcifications were expressions of degenerative alterations Considering this divergence of opinions as regards the nature of thyroid calcifications we have found it might be of interest to study a thyroid material originating from a series of autopsies of persons over 40 Histological and biophysical methods have been applied in our study

MATERIAL AND METHODS

s over 40 years of age
artment of Pathology
the average age being
14 years) In all cases but one no clinical signs could be seen of any disturbance in the thyr
out to be a case of

e mean weight was
aldehyde for 1 to 4
a focus of 0.3 mm
n film and focus being 2 metres so
re We used Kodak Crystallex In
DX 80 This film has a maximum
10–20 lines/mm which makes it possible to obtain pictures of calcifications of less than 1 mm in diameter

The photographed calcifications were dissected and divided into two even parts

This study has been aided by grants from *Sigrid Juselius Stiftelse* and the *Finnish Medical Council*

for histological and microradiographic preparation respectively The part to be *histologically* examined was calcified, dehydrated with alcohol via xylol embedded in paraffin and cut into slices of a thickness of 5 6 μ These were stained with haematoxylin eosin and haematoxylin-Van Gieson The part to be *microradiographed* was dehydrated with alcohol, embedded in methylmetachrylate and cut into thin slices, which were ground down to a thickness of 75–100 μ and microradiographed according to the conventional technique (*Engstrom* 1956) Kodak Spectroscopic Plates (No 649) were used as photographic material, these being exposed with 15 40 kV and 10 mA In two cases the thyroid calcifications were so fine that histologically thin slices of them were exposed in ultrasoft X rays, whereby details down to cell level could be distinguished (*Engstrom* 1956)

The methylmetachrylate embedded and ground preparations from 15 cases were analysed also with regard to their chemical composion by means of a microdiffraction camera, which enables separate analyses to be made of areas of a diameter of 20 μ (*Chesley* 1947) This technique provides information concerning the chemical composition and, furthermore, of the size of crystallites and the orientation of crystals in the preparation (*Carlstrom* 1955) The microradiogram obtained previously was used at the point analyses when the areas suitable for analysis were chosen Various point analyses were carried out with all 15 preparations

RESULTS

By means of this technique calcifications were registered in 25 out of the 101 cases These were isolated in 14 cases and multiple in 11 cases In 4 cases the largest diameter was less than 1 mm, in 13 cases 1 to 5 mm, and in 8 cases more than 5 mm



Fig 1

Calcified thyroid gland Below and to the left hyalinized and calcified connective tissue with atrophic follicles Five giant follicles are present Upwards to the left normal gland tissues Haematoxylin and Van Gieson staining Magnification 15 ×



Fig 2
Calcified focus from thyroid gland showing metaplastic bone and bone marrow Haematoxylin and Van Gieson staining Magnification 50 ×

Histology The microscopic examination indicated that distinct alterations of a degenerative nature had taken place in the calcified areas In these areas only few follicles could be formed which were greatly enlarged and filled with a homogeneous colloid substance The epithelium in follicles was low, and in some places it did not exist at all Between the enlarged follicles plenty of altered connective tissue was to be found which consisted of hyalinized collagenic fibres Between these fibres there were often atrophic follicles without colloidal contents (Fig 1) In five cases metaplastic bone and marrowlike tissue could be seen near the hyalinized fibres (Fig 2) In the two cases where ultrasoft X-rays were used it became evident that the mineral salt had deposited in the connective tissue between follicles (Fig 3) The non-calcified areas consisted of normal thyroid tissue

In the one case of cancer the thyroid was enlarged (58 grammes), and the cancer was a giant cell carcinoma The gland contained various great calcifications These were not located in cancer tissue, on the contrary, they were on the other side The calcified areas showed degenerative alterations of the same value as the ones seen in other cases containing calcifications The remaining 24 cases showed no other pathological alterations than those described above

Microradiography The microradiogram indicated that all the calcifications were of the same kind as far as their structure was concerned



Fig 3

Ultra soft microradiogram from a slightly calcified thyroid Normal follicles and connective tissue are seen in the lower part of the picture Upwards to the left the white areas between the follicles are calcium salts deposited in connective tissue Exposed at 1 5 A Magnification 30 ×

They were very radio-opaque, and had often the form of round cysts These were most opaque in the periphery, while the central parts were only slightly calcified or did not present any mineral salt deposits at all The structure of the calcified parts was often diffuse Occasionally however, structures of concentric or parallel fibres could be seen Some preparations presented small areas filled with small, oblong, crystal like, radio-opaque bodies (Fig 4)

Roentgen diffraction All the analyses indicated that the calcifications only contained hydroxylapatite $Ca_{10}(PO_4)_6(OH)_2$ and that the size of crystallites in the analysed areas did not vary The crystallites were of the same size as the apatite which forms the inorganic component of bone tissue No orientation of crystallites could be noticed

DISCUSSION

This study has shown that calcifications in the thyroid gland are relatively common occurrences in persons over 40 Among the 101 examined cases no less than 25 had calcified areas in the thyroid gland, and in 8 cases calcifications were very large and might have been visible by roentgen-diagnostic examination of the soft parts of neck Certain scientists (*Holtz & Powers* 1958, *Gerard-Marchant et al* 1962)



Fig 4

Microradiogram from a thyroid calcification Calcium salts are heavily deposited and show diffuse arrangement The black rings in the picture indicate areas where the diffraction analyses have been made These showed hydroxylapatite pattern Exposed at 50 Å Magnification 15 ×

have been of the opinion that calcifications in thyroid glands, visible in roentgenogram, would indicate occurrence of cancer Our findings are in contradiction with this conception and indicate, in addition, that calcifications are located in degeneratively altered tissue Even in the one case of thyroid cancer, the calcifications were not situated in cancer tissue their location being a degeneratively altered area The opinion that calcifications might be an expression of adenoma development can be disproved, too, as adenoma existed in none of our cases Our study has shown that thyroid calcifications are expressions of degenerative alterations and are of no diagnostic value

It is an old discovery that a deposit of calcium salts takes place in degeneratively altered tissues of the body A number of crystallographic examinations of different calcifications (*Brandenberger & Schinz* 1945) has indicated that the deposited calcium salt is hydroxylapatite $Ca_{10}(PO_4)_6(OH)_2$ All the calcifications in our material also consisted of hydroxylapatite Our microradiographic examinations showed that the calcified areas were lacking the char...

tions was the same as that described in reports on other similar calcifi cations and bone (*Carlstrom & Glas* 1959) In normal bone the apatite crystallites are located in certain directions depending on the collagen component This can be seen on the diffractogram The diffractograms of our analyses show that the apatite in thyroid calcifications is not orientated, which indicates that the tissue which forms matrix for the deposits, is not regularly arranged

SUMMARY

Autopsy preparations of 101 thyroid glands from persons over 40 years of age were examined radiologically, histologically and microradiographically with regard to calcifications In 25 cases calcifications were found and they all consisted of hydroxylapatite They are due to degenerative changes, and are of no roentgendiagnostic value

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The Department of Pathology Section II University of Helsinki

ON INTRATUBULAR CALCIFICATION IN THE EPIDIDYMIS

By

JOHAN AHLQVIST ARTO LAHTIHARJU and GUSTAF ELFVING

Received 11 viii 65

In a biopsy specimen from the epididymal head recently studied in our laboratory rather heavy intratubular calcifications were observed Since such calcifications *are not mentioned* in the usual textbooks on pathology nor known to the pathologists whom we have consulted it seemed worth while to discuss the question in spite of that at least small calcified bodies do not seem to be too uncommon (*Oberndorfer* 1931) Small calcifications were found in 2 further cases out of the 31 specimens from the epididymis studied in our laboratory during the last 5 years

OBSERVATIONS

Case I (T 2854 64) A male 43 years old for one year had been treated with tuberculostatics because of tuberculosis of the left kidney and of the left epididymis The right epididymis later became swollen and was removed because tuberculosis was suspected

Histologically many of the tubules were dilated and retention of sperm was present Intratubular calcifications were present in different areas (Fig 1) The

ry reaction but no tuberculosis The calcified bodies formed violet lakes with Mayer's hemalum
alizarin Red S acc
metachromatic wit
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solution of hydrokinon In other parts of the tubuli there among others were found agglutinated and degenerated sperm cells without signs of calcification Some agglutinates of sperm cells seemed to be lying outside the epithelium

Further cases In a case (T 392 59) of fibrosis of the epididymis of unknown origin retented sperm and agglutinates of sperm cells gave weak histochemical reactions for calcium Small both extra and intratubular calcified bodies were present in otherwise healthy parts of the organ in a case (T 1424 63) of granulomatous epididymitis.

[1] Aided by a grant from the *Sigrid Juselius Foundation*

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[1] Aided by a grant from the *Sigrid J* [illegible]

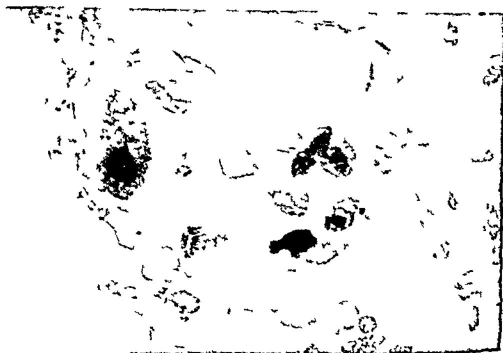


Fig 1
Area containing intratubular calcified bodies which stained orange red with alizarin Red S No counterstain was used × 48

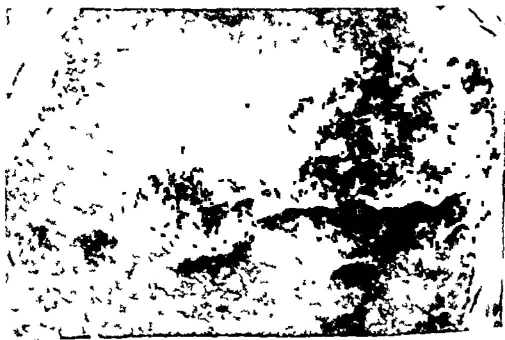


Fig 2
In this tubule the calcified bodies had a granular appearance and stained violet Mayers hemalum × 480



Fig 3
The rather homogenous area in the right part of the tubule formed violet lakes with hemalum while the rest of the stained tubular contents were blue × 480

DISCUSSION

Small agglutinates of sperm cells are fairly common in the epididymis These often contain large nuclei but we are not aware of whether these should regarded as signs of phagocytosis of sperm cells (*Wegelin* 1921) or whether they are masses of cells desquamated from the testis (*Stiepe* 1925, *Biasi* 1929), whether one should talk about spermiophages or pseudospermiophages as does *Oberndorfer* (1931) These agglutinates are often seen in cases of retention of sperm According to Oberndorfer they may undergo necrosis and subsequently calcify In a case of catarrhal inflammation he describes calcified desquamated epithelial cells lying among pseudospermiophages

In our first case the positive von Kossa reaction indicates that calcium probably was present in the form of carbonates or phosphates, on the other hand it is known that dried bovine sperm contains about 12 per cent lipids as already shown by *Kolliker* (1856), it is thus possible that calcium soaps may have been present too According to the monograph of *Mann* (1964) the duration of the passage of sperm through epididymis seems to be much longer than generally recognized It seems, moreover that the epididymis is the major site of resorption of non-ejaculated sperm but the mode of this resorption is said to remain unknown (*Amann & Almquist* 1962) in spite of that various mechanisms were presented in the review by *Oberndorfer* in 1931 According to Konigstein (1908) sperm cells may dissolve in the seminal vesicle and

Oberndorfer believes that the small agglutinates described above may dissolve in the epididymis, since he never saw them in the tail of the organ. Many others have observed sperm cells outside the tubular epithelium, especially in various kinds of inflammations. Such ones may have caused the extratubular calcifications present in the last case.

The functional significance of these calcifications probably is fairly small.

SUMMARY

Intratubular calcifications of the epididymis apparently formed by degenerated sperm cells are described and discussed.

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TRIC (TRACHOMA-INCLUSION CONJUNCTIVITIS) AGENTS AND INTERFERON

By

C H Mordhorst and V Reinicke

Received 17 v 65

A large number of viruses has been shown to induce a production of interferon when inoculated into cell systems derived from a variety of species (13) Isaacs has therefore suggested that production of interferon is a common response of vertebrate cells to viral infections (13) However, considerable variation in interferon inducing ability has been found for different viruses, even when tested under the same experimental conditions (8, 9, 10, 12, 14, 25, 26, 28, 30) A considerable variation in their sensitivity to the antiviral action of interferon has also been reported and evidence has accumulated that a certain relationship may exist between the potency of a virus as an interferon inducer and the sensitivity of the virus to interferon in the sense that high interferon inducing capacity is often correlated with a pronounced sensitivity to interferon (9, 14 26, 30)

A group of agents which, to our knowledge, has not been associated with production of interferon is the psittacosis lymphogranuloma venereum trachoma group This group of organisms presents an interesting and controversial problem in taxonomy (1, 2, 4) Although obligate intracellular parasites of a size approximating certain viruses these micro organisms have developmental and biochemical properties that appear to have much in common with the bacteria and rickettsias However the issue is by no means settled, and there is increasing support for the view expressed by *Weiss* (31) that this group of organisms is in fact typical of neither rickettsias nor viruses, but *is placed somewhere between the two*

The finding that tissue cultures infected with rickettsia tsutsugamushi produced interferon (11) together with the finding that certain viruses were able to induce interferon production by intravenous injection into mice (3) and chicks (32) prompted the present study where interactions between the trachoma inclusion conjunctivitis (TRIC) agents and interferon were investigated

The authors wish to thank Miss *E Christensen* and Mrs *B Saugbjerg* for skilled technical assistance

MATERIALS AND METHODS

Viruses The following TRIC agents of trachoma and inclusion conjunctivitis were used ASGH[1], BOUR, SA 2 T ang TL 55 and SS 1[2]

The titre (50 per cent egg lethal doses (LLD50)) and yolk sac passage (ys) of the employed strains are recorded in connection with the respective experiments

For preparation of Interferon batches, Influenza A Melbourne and Influenza A WS were employed These two strains were both in the 7th to 9th egg passage in this laboratory and had titres of about 109.0 LID50/ml Passage and storage of these strains have been described previously (20) The attenuated strain of poliovirus type 1, LSc 2ab[3] was employed for titration of monkey interferon

Eggs 6 to 8 day old white Leghorn eggs were used for the preparation and titration of TRIC agents and 10 to 11 day old eggs for the preparation of influenza virus and production of interferon

Technique of TRIC agent cultivation ... age and infectivity titrations has been de...

Semipurified suspensions of live TRIC ... low and high speed centrifugation A 50 ... 2 000 r p m./5 min in a conventional low ... layer containing the elementary body pa... spun at 7 000 r p m (approximately 4 000G) for 45 minutes in a Spinco model L ultracentrifuge The supernatant was discarded and the sediment was resuspended in the original volume of phosphate buffer at pH 7 38 and submitted to one more cycle of high speed centrifugation The resuspended sediment from the second high speed centrifugation constituted the partially purified virus suspension *Purified* suspensions of various TRIC agents were obtained from heavily infected yolk sac membranes by treatment with fluorocarbon[4] according to the method described by *Bernkopf* (5) This purification procedure destroyed all infectivity for the embryonated egg The purified preparations were temporarily stored at —20° C and before use adjusted in a phosphate buffer solution to contain approximately 1×10^9 particles per ml as determined by direct count (19)

Inoculation of animals 1 Monkeys In the present experiment a total of 10 Cercopithecus aethiops monkeys was included Previous experiments in this laboratory have shown that Cercopithecus monkeys are as susceptible to infection with various TRIC agents as are Rhesus and ... method of conjunctival inoculation of monkeys wi... clinical signs cytologic examination and counting ... described previously (16)

Blood specimens were collected immediately before ocular infection and convalescent sera were obtained 3 and 5 weeks later The sera were titrated for group specific complement fixing antibodies as previously described (17)

2 Roosters 12 one year old white Leghorn roosters were used in attempts to obtain a production of interferon by intravenous injection of high titre TRIC agent suspensions into the animals Each animal was given a single intravenous injection of between 0 25 ml and 1 0 ml of virus Blood samples were drawn by heart puncture 3, 6 16 and 24 hours after the intravenous injection

Preparation of interferon batches For experiments in embryonated eggs a batch of *egg interferon* was obtained from 10 to 11 day old eggs which had been inoculated

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... Institute for trachoma and inclusion ...

... ang et al (29) after an unknown number of yolk sac passages)

2 The ASGH, BOUR and T ang strains were kindly supplied by Miss L Hanna San Francisco and the SA 2 strain was kindly supplied by Dr L H Collier Lister Institute, London

3 Kindly supplied by Dr A Sabin

4 Freon 113 (Trichlorotrifluoroethane) Du Pont de Nemours and Co Inc

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bi ..
magglutinating capacity The interferon was lyophilized in 10 ml amounts and stor ... at +4° C When resuspended in Earle's BSS the interferon batch was found
I

following was Confluent sheets of Cercopithecus monkey kidney cells grown in
]

continued at 36° C The tubes were read microscopically twice daily and the final reading was made when the control tubes showed 85–100 per cent degeneration usually after three days The titre of interferon was expressed as the dilution of interferon which protected 50 per cent of the cell sheet from showing cytopathogenic effects By this technique the interferon batch had a titre of 1 32 The resuspended control batch had no interfering capacity

Titration of sera for interferon activity Sera from Roosters were titrated for content of interferon by using the previously described Sindbis plaque inhibition system (22 23) with the modification that pre incubation for 20 hours instead of 3 hours was employed

EXPERIMENTAL

In the first series of experiments the influence of interferon upon the growth of TRIC agents in the yolk sac of embryonated eggs was investigated The trachoma strain ASGH (ys 17) and the inclusion blennorrhoea strain SS 1 (ys 15) were titrated employing virus dilutions of 10^{3} to 10 and groups of 7 eggs per dilution Lyophilized egg interferon resuspended in 1/10 the original volume was added in identical amounts to the tubes of the dilutions series Four titrations were performed and the amount of interferon employed per egg through the experimental series was found to vary between 0 045 ml and 0 21 ml of the tenfold concentrated suspension

The results appear from Table 1 and Fig 1 Table 1 shows that the final titres (ELD 50 ml) of the two TRIC agent strains obtained in interferon treated chick embryos were not significantly different from those of the controls Fig 1 demonstrates the dose response curves for two representative titrations of the ASGH and SS-1 strain, respectively The arithmetic mean of the death time in days for each group of chick embryos is plotted against the dose (log_{10}) of inoculated TRIC

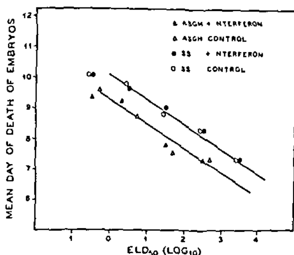


Fig 1

Dose response curves for a trachoma (ASGH) and an inclusion blennorrhoea strain (SS 1) in chick embryos treated with interferon

agent It can be seen that the log_{10} dose is inversely proportional to the mean death time for the two strains and that the growth rate of the TRIC agents as well as the final infectivity titres were the same whether interferon was present or not Complete insensitivity to interferon was thus encountered for the employed TRIC agents in this test system

TABLE 1

Titres of a Trachoma Strain (ASGH) and an Inclusion Blennorrhoea Strain (SS 1) Obtained in Chick Embryos Treated with Interferon

Exp No	TRIC strain	Passage no	Interferon (ml/egg of 10 × conc susp)	Titres (ELD50/ml) log_{10}
I	ASGH	17	0 045	6 8
	ASGH	17	Control	7 0
II	ASGH	17	0 190	6 3
	ASGH	17	Control	6 5
III	ASGH	17	0 200	6 5
	ASGH	17	Control	6 7
IV	SS 1	15	0 210	6 5
	SS 1	15	Control	6 3

In the following experiment the effect of interferon upon the growth of a TRIC agent in the conjunctiva of Cercopithecus monkeys was investigated To this end 10 monkeys were infected conjunctivally in both eyes with the inclusion blennorrhoea strain SS 1, each eye receiving approximately $10^{4.3}$ ELD50 During a 12-hour-period prior to ocular infection and for an 18 hour-period immediately after, half of the

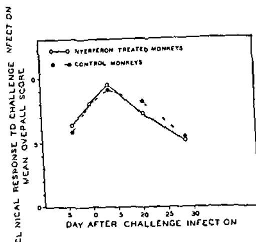


Fig 2

Average response of interferon treated monkey eyes to challenge infection with SS 1 YS 6 $10^{4.3}$ *ELD50*

animals were treated every hour with monkey interferon instilled as eye drops into the conjunctival sac The remaining 5 monkeys were similarly treated with a control fluid suspension Interferon as well as control fluid were marked in code so that the experiment was performed *according to double blind test technique* The average clinical response to ocular challenge infection in both groups of monkeys is illustrated in Fig 2 All animals developed typical, although mild, conjunctival inflammation most pronounced during the first 3 weeks and subsiding gradually over the following week No difference between the interferon treated and control groups of animals was observed throughout the course of infection and both groups of animals developed complement fixing serum antibodies of the same order during the convalescent period Again, complete insensitivity to the action of interferon was encountered for the employed TRIC agent

The preceding experiments were concerned with the influence of preformed interferon upon the growth of TRIC agents In the subsequent experiments the ability of TRIC agents to induce production of interferon *in embryonated eggs as well as in roosters* was studied

A group of 60 seven day old eggs were inoculated by the yolk sac route with the inclusion blennorrhoae strain SS-1, each egg receiving approximately $10^{4.0}$ LD50 estimated to kill 50 per cent of the inoculated eggs after 7 days of incubation at 35° C When the eggs had been incubated for 7 days they were divided into two groups containing dead and surviving embryos, respectively From a minimum of 8–9 eggs pools were made of yolk sac membranes, allantoic fluids and amniotic fluids separately from live and dead embryos Control materials were obtained from 13-day old non inoculated eggs The harvested materials

were inactivated for half an hour at 56° C to destroy the viral activity (24). Following inactivation the harvests were tested for interferon as previously described and it was found that none of the examined materials contained interferon. Obviously the TRIC agent during a growth period of 7 days in embryonated eggs did not induce production of interferon.

TABLE 2

Interferon Titrations of Sera from Roosters Inoculated with Varying Amounts of Different TRIC Agent Strains

Exp No	TRIC strain	Titre (log_{10})	Amount inocul (ml)	Animal	No of Plaques* obtained in 1:8 dilutions of sera collected at hours after inoculation			
					3	6	12	24
I	ASGH	7.0	0.25	1		65.5	65.0	73.0
			1.0	2		69.0	70.5	83.0
			1.0	Control			65.5	
II	T'ANG II 55	7.1	0.25	1	50.0	59.5	63.0	57.0
			0.25	2	50.0	42.0	55.5	51.0
			1.0	Control			58.0	
III	SA 2	7.5	0.25	1	55.5	62.0	63.0	59.5
			0.75	2	67.0	64.5	73.0	63.5
			1.0	Control			63.0	
IV	Fluorocarbon treated BOUR	9.3	1.0	1	43.5	51.0	48.0	41.5
	Fluorocarbon treated SS 1	9.0	1.0	2	52.0	60.0	56.0	55.5
			1.0	Control			53.0	

* Average of three cultures

In a final series of experiments attempts were made to produce interferon in roosters by intravenous injection of TRIC agent suspensions. The strains of ASGH, SA-2 and T'ang were used as live yolk sac suspensions and the strains BOUR and SS-1, as non-infectious fluorocarbon purified suspensions. In the first experiments the animals received 50 per cent crude yolk sac suspensions intravenously, but since this procedure caused an excessive lethality under the clinical picture of acute fat embolism, the experimental technique was slightly changed in the subsequent experiments so that these were performed with partly purified stock suspensions. Control animals received either non-treated, partly purified or fluorocarbon treated suspensions obtained from non-inoculated eggs. Purification of virus and use of only small intravenous inocula (i.e. $\leqq$ 1.0 ml) diminished, but did not abolish death amongst the experimental animals. The animals were bled at 3, 6, 12, and 24 hours after inoculation of virus and the sera were tested for content of interferon. The results can be seen in Table

2. No inhibitory capacity was observed in any of the 1:8 diluted sera regardless of the type of TRIC agent employed, the size of inoculum and the time for obtaining the serum samples. Many of the sera were also tested diluted 1:4 and 1:16 and plaque counts were found to be the same as those obtained with 1:8 diluted sera. Thus also animal inoculation failed to show interferon inducing capacity of the employed TRIC agent strains.

DISCUSSION

The present experiments failed to show any inhibitory effect of interferon on the growth of TRIC agents in embryonated eggs. The possibility that the lack of effect might be due to a use of insufficient amounts of interferon is unlikely since previous studies have shown that amounts of interferon similar to those employed in the present experiments have a marked inhibitory effect on influenza virus growth in ovo (21).

In addition the present experiments showed that treatment of monkey eyes with interferon consisting of repeated instillations of interferon onto conjunctival epithelial cells, did not inhibit the growth of the TRIC agent. In contrast, vaccinia virus grown in the epithelial cells of the eye is known to be easily inhibited by topical instillation of interferon (7, 15). It seems therefore reasonable to conclude that TRIC agents are insensitive to the action of interferon.

TRIC agents did not induce formation of interferon neither when grown in embryonated eggs nor following intravenous injection in roosters. In the latter experiments care was taken to employ high titred suspensions of TRIC agents because it has been shown that viruses induce formation of interferon only when administered intravenously in large quantities (3). The failure to demonstrate any formation of interferon with the TRIC agents also under these conditions indicates that these agents lack the ability to produce interferon at least in the host systems studied.

Since interferon production and sensitivity to interferon seem to be a common feature of viruses (13) the present findings support the view that TRIC agents are microorganisms which do not belong in the group of proper viruses.

SUMMARY

The present study has shown that interferon does not inhibit the growth of TRIC agents in embryonated eggs or in monkey eyes. It has furthermore been demonstrated that TRIC agents lack the ability to induce formation of interferon during growth in embryonated eggs or following intravenous injection of high titred suspensions into roosters. The possible role of the present findings for the classification of TRIC agents is briefly discussed.

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of 40–210 plaques

Some, but not all experiments revealed a normal distribution of plaque diameters However a coefficient of variation of 40 per cent was found in all instances and it was therefore considered *justifiable to compare mean* values according to the formula

$$u = \frac{\bar{x}_1 - \bar{x}_2}{0.4\sqrt{\frac{\bar{x}_1^2}{n_1} + \frac{\bar{x}_2^2}{n_2}}}$$

where u is approximately normally distributed with a mean value 0 and a standard deviation 1

Media Growth medium, maintenance medium and overlay medium were identical with those previously described (12, 13, 14)

Hormones Pure crystalline preparations of metandienonum testosterone and oestradiol were dissolved in minute amounts of 99 9 per cent ethanol after which they were mixed with maintenance medium The biologically non active steroids

trate hydrochloride buffer, respectively

Tissue culture Chick embryo fibroblast (c e f) tissue cultures grown in plastic Petri dishes (diameter 4 5 cm) as previously described were employed (12, 13)

EXPERIMENTAL

The first experiment was concerned with the effect of 24 hours' pre-incubation with varying doses of metandienonum upon development of size of Sindbis virus plaques in c e f cultures Six groups each consisting of 12 confluent monolayer cultures of c e f cells had their growth medium removed and replaced with medium containing metandienonum in concentrations ranging from 0 016 γ ml to 10 γ/ml A control group received control medium only After 24 hours of incubation at 36° C the medium was removed and the cultures were inoculated with approximately 20 p f u of Sindbis virus and incubated for one more hour at 36° C The cultures were then overlayed and replaced in the incubator Five cultures from each group were removed after 24, 48 and 72 hours of incubation and the plaques were measured In accordance with previous results (1) no significant differences (10, 12) between number of plaques appearing in the hormone treated cultures and in the control cultures were observed But as appears from Table 1 metandienonum was found to have a marked enhancing effect on the size of the Sindbis virus plaques The effect was most marked when a concentration of 10 γ metandienonum ml was employed, less pronounced

[1] Obtained from Fluka AG, Buchs SG, Switzerland.

[2] Obtained from E Merck AG, Darmstadt, Germany

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INCREASE OF SIZE OF SINDBIS VIRUS PLAQUES DUE TO THE EFFECT OF STEROID HORMONES

By

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Received 11 vii 65

The size and number of virus plaques obtained on a cell monolayer under a solidified overlay has been shown to be influenced by a number of factors (1, 2, 4, 11) The type of cells and virus employed and also the properties of the overlay play a rôle in this connection Increasing depth of the overlay, as well as the presence of inhibitory substances in certain overlays decrease the number and size of plaques of many viruses (1, 4) Recent experiments by *De Maeyer & De Maeyer* have shown that treatment of rat embryo tissue cultures with carcinogens increased the size of Sindbis virus plaques in the cultures (3, 5, 7) These authors suggest that the plaque size enhancing effect of the carcinogens could be due to inhibition of interferon production during plaque development (3, 5, 7)

It seemed of interest therefore to investigate whether steroid hormones might similarly enhance the size of Sindbis virus plaques, since it has been shown that a number of these hormones are able to reduce the production of interferon in chick embryo fibroblast (c e f) cultures infected with influenza virus (13, 14) Three different steroid hormones, metandienonum, testosterone and oestradiol and two steroids, pyrene and anthracene, without hormonal or carcinogenic properties were employed in the present experiments A pituitary growth hormone with *in vivo* effects reminding of certain steroid hormones (8, 9) was also included in the study

MATERIALS AND METHODS

Most materials and methods have been described in detail elsewhere (12 13 14)

Virus A plaque purified clone of Sindbis virus strain Egypt AR 339 (kindly submitted by Dr E De Maeyer Rega Institute Louvain Belgium) was employed This clone is a large plaque variant twice purified in rat embryo cells under agar and it was ch [illegible] shown to be insensitive to the [illegible] nt in agar overlays (7) In this [illegible] ne passage in c e f cells

In m [illegible] rus were inoculated into

The author wishes to thank S *Olesen Larsen* M A (econ) for performing the statistical analysis and Mrs *H Saugbjerg* for skilled technical assistance

que diameters calculated from plaque measurements 24, 48 and 72 hours after virus inoculation were found to be the same in pyrene, anthracene and growth hormone treated cultures as in the control cultures.

DISCUSSION

The present study shows that the steroid hormones metandienonum, testosterone and oestradiol all have an enhancing effect on the size of Sindbis virus plaques in c.e.f. cultures while the steroids pyrene and ... effect

pyrene, anthracene and growth hormone had no influence on interferon production (14, 15) seem to indicate a correlation between increase of plaque size and decrease of interferon production. The fact that testosterone increased plaque size to a greater extent than did oestradiol also support this hypothesis since it has been shown that testosterone has a considerably greater inhibiting effect on interferon production than has oestradiol (14).

The present findings are in accordance with the observation by *De Maeyer & De Maeyer* on the plaque size enhancing effect in rat embryo cultures of carcinogens and ultraviolet radiation, both of which also reduce the synthesis of interferon (3, 5, 6). Some previous experiments performed in this laboratory might have led to the observation of a plaque size enhancing effect of steroid hormones in c.e.f. cultures (12). However, the phenomenon was not observed at that time. There are several explanations for this. 1) Only a rather limited number of experiments which could have revealed the phenomenon was performed. 2) Plaques were routinely read after 48 hours incubation, while the present findings indicate that the phenomenon in most cases becomes more marked on prolonged incubation. 3) The phenomenon is considerably less pronounced in c.e.f. cells than in rat embryo cells (7). 4) A noncloned strain of Sindbis virus was employed, and the phenomenon is more easily observed with a purified virus strain.

SUMMARY

Preincubation for 24 hours with 20 γ/ml of the steroid hormones metandienonum, testosterone and oestradiol was found to increase the size of Sindbis virus plaques in c.e.f. cultures. The plaque size enhancing effect was considerably more pronounced when metandienonum and testosterone instead of oestradiol were employed. With the present technique the minimal plaque size enhancing concentration of metandienonum was found to be between 2 γ/ml and 0.4 γ/ml. It is suggested that the plaque size enhancing effect is correlated to the reducing effect on interferon synthesis of the steroid hormones. This hypo-

with 2 γ metandienonum/ml and it disappeared at lower concentrations After preincubation with 10 γ/ml the plaque size enhancing effect became more pronounced with time, while a further increase, beyond that of the controls, was not observed after 48 hours of incubation when preincubation with 2 γ/ml had been employed

TABLE 1

The Influence of 24 Hours' Preincubation with Varying Doses of Metandienonum on the Size of Sindbis Virus Plaques in c e f Cultures

Concentration of metandienonum employed for preincubation (γ/ml)	Average plaque diameters (mm)* hours after inoculation 24	48	72
10	0 8 §	1 7 § †	2 8 § †
2	0 7 §	1 2 § †	1 8 § †
0 4	0 5	1 1 §	1 6
0 08	0 5	1 0	1 3
0 016	0 4	0 7	1 3
Control	0 5	0 9	1 5

* Measurements performed on 18 33 plaques were employed for calculation of each

§

†

TABLE 2

The Influence of 24 Hours Preincubation with 20 γ/ml of Different Hormones on the Size of Sindbis Virus Plaques in c e f Cultures

Hormone	Average plaque diameters (mm)* hours after inoculation 24	48	72
Testosterone	0 6	2 3 § †	2 4 § †
Oestradiol	0 5	1 3 †	1 8 § †
Control	0 5	1 2	1 4

* Measurements performed on 21 40 plaques were employed for calculation of each average diameter
For other signatures See Table 1

In the following experiment the effect of 24 hours' preincubation with 20 γ/ml of testosterone and oestradiol was investigated The experimental technique was similar to that described above As shown in Table 2, testosterone as well as oestradiol enhanced the diameters of the virus plaques, the effect of testosterone being considerably more marked than that of oestradiol

The subsequent two experiments were concerned with the effect on plaque size of two biologically inactive steroids, pyrene and inthracene as well as of pituitary growth hormone The technique employed was the same as that described for testosterone and oestradiol Average pla-

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SPECIFICITY OF ANTI-ANTIBODIES IN HUMAN SERA

By

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Different groups of factors in human sera behave as antibodies directed against γG globulin originating from various species including man These factors are usually γM-globulins and are detectable by red cells sensitized with γG-globulins In human sera a 22S complex formed through interaction between γG- and γM globulin molecules has been demonstrated (4, 19)

The heterospecific factors (rheumatoid factors) are directed against γG globulin from rabbits or other animals and are usually inhibited by *denatured human γG globulin but not by native human γG globulin* (for references see 27) Experimental observations indicate that autologous γG-globulin may be the stimulus for production of rheumatoid factors (3)

The anti Gm and anti-Inv factors are directed against genetically determined structures of the heavy and light polypeptide chains of the antibody molecule respectively (8) These factors are easily inhibited by native human γ globulin of the corresponding Gm or Inv type Anti Gm factors originating from normal persons (Snagg anti-Gm factors (5)) show isospecificity and may result from isoimmunization (1, 6) Anti Gm factors in sera from patients with rheumatoid arthritis (Ragg anti Gm factors (5)) also show isospecificity but differ in some respects from the Snagg anti Gm factors However, zonal phenomena in agglutination of sensitized red cells by some Ragg anti Gm sera have been attributed to the occurrence of autospecific anti Gm factors (13) Iso- and autospecificity of anti Gm factors are still discussed (5)

Factors directed against hidden antigenic determinants have been demonstrated by agglutination of red cells sensitized by some split products of the γG globulin molecules (16)

Another group of anti- γG globulin factors was described by *Milgrom et al* (23) who called it anti antibody and by *Grubb* (10) who called it non rheumatoid arthritic type agglutinator, (here referred to as anti-antibody) (for further references see 22) Anti-antibodies are directed

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thesis is in agreement with the finding that the steroids pyrene and anthracene as well as pituitary growth hormone which do not reduce the synthesis of interferon are without any effect on the size of Sindbis virus plaques

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ethanol (Fluka AG, Buchs, Switzerland) were mixed and kept over night at room temperature The mixtures were then tested for various activities Proper controls

[illegible]
controls were included

Gm typing was performed as previously described (26)

EXPERIMENTS AND RESULTS

Anti γ Globulin Factors Detected

In 5 out of the 14 sera with anti-antibody activity heterospecific rheumatoid factor was detected (Table 1) In one serum also an anti-Gm factor was present (Table 2) The heterospecific rheumatoid factor was absorbed by sheep red cells sensitized by rabbit antiserum Red cells sensitized by anti-D 3083 absorbed the anti-antibody Anti-D 3091 sensitized red cells absorbed the anti Gm factor Anti antibody was absorbed leaving the heterospecific rheumatoid factor and vice versa (Table 1) From serum 355 each of the three anti-γG globulin factors was absorbed independently leaving the activities of the 2 others (Table 2)

TABLE 1

Titres of Anti Antibody Sera before and after Absorption with Red Cells Sensitized by Rabbit or Human γG Globulin

Anti antibody serum	Absorption with red cells					
	unsensitized		sensitized by rabbit antibody		sensitized by anti D 3083	
	Waaler Rose test	Anti D 3083 test	Waaler Rose test	Anti D 3083 test	Waaler Rose test	Anti D 3083 test
55	128	64	< 8	64	128	< 2
355	512	32	< 8	32	256	< 2
647	64	32	< 8	32	64	< 2
648	129	64	< 8	64	128	< 2
649	16	16	< 8	16	16	< 2

Controls The respective unsensitized red cells and the anti antibody sera in dilutions no agglutination The respective sensitized red cells and saline no agglutination

The specificity of the anti Gm factor of serum 355 absorbed of anti-antibody was tested in two-dimensional titrations (11) The anti Gm factor was strongly inhibited by Gm(a+x+) and Gm(a+x—) but not by Gm(a x) serum This indicated that serum 355 contained anti-Gm(a) in addition to the anti-antibody and the heterospecific rheumatoid factor

Reduction with mercaptoethanol abolished the agglutinating activities of the anti-antibodies, the anti-Gm(a) factor and the heterospecific

against isospecific γG-globulin acting as antibody in an antigen antibody (immune) complex and are not inhibited by native γG globulin (16, 23) *Milgrom et al* (23) found equally strong reaction against red cells sensitized by various potent incomplete antibodies However, *Andresen* (2) has briefly reported that most of his anti-antibodies did not agglutinate red cells sensitized by γG-globulin of every type

This paper presents observations on 1 the molecular basis for different groups of anti- γG-globulin factors, 2 specificity of anti-antibodies in agglutination of red cells sensitized by anti-D antibodies with different Gm type

MATERIALS AND METHODS

Red cells O, R_1R_2 (CDe cDE) red cells from a single donor were stored at 4° C in acid citrate dextrose solution for maximum 5 days Sheep red cells were handled and stored as described by *Tonder* (28 p 20)

Anti Gm sera incomplete anti D sera and normal sera All sera were used in earlier investigations (26) except some sera in the panel of anti D antibodies These were however incomplete antibodies selected as previously (26)

Anti antibody sera A panel of 875 sera mainly from patients in *Hallestad Hospital* and in *Bergen University Hospital* was examined for anti γG globulin factors The sera were tested in dilutions 1 4 1 16 and 1 64 against red cells sensitized by anti D sera specific for Gm(a) Gm(x) Gm(b) and Gm(f) Seven sera with anti antibody activity were found Six other sera were kindly provided by Dr *M Harboe* Five of them were designated by the letters used by him (16) Some new samples of these sera showed a lower titre than originally Data on the 6th serum (649) have not previously been published A new serum sample (355) from the patient L H (11 15) was provided through the courtesy of Dr *M Harboe* Accordingly 14 anti antibody sera were available

Anti human gammaglobulin serum Antiserum was obtained after immunization of albino rabbits by intramuscular injections of human serum in complete *Freund's* adjuvant

Rabbit anti sheep red cell serum Antiserum was produced as described by *Tonder* (28 p 21)

Gammaglobulin ion, batch 80779 was kindly provided by

Agglutination test of incomplete anti D

Agglutinating activity of anti antibodies was tested by the tube technique Gm typing was performed by the slide technique

The *Waaler Rose* test was performed according to a slight modification of the method described by *Tonder* (28) Serum was absorbed by unsensitized sheep red cells and titrations were made in 0 1 ml volumes An equal volume of 0 5 per cent sheep red cells sensitized by 1/3 agglutinating unit of anti sheep red cell serum was added to each tube of diluted serum The tubes were shaken and left at 4° C over night Readings were made without centrifugation and graded as described for the tube technique

Absorption experiments For absorption with anti D sensitized red cells equal volumes of packed sensitized red cells and antiserum diluted 1 2 or 1 4 were mixed The mixtures were left at room temperature for 2 hours One absorption was usually sufficient to remove the anti antibody or anti Gm activities in a given serum while 4 absorptions were used to absorb the anti Gm factor in serum 355

For absorption with sensitized sheep red cells one ml of serum diluted 1 8 was absorbed twice by 0 5 ml of washed packed sheep red cells sensitized by 10 agglutinating units of anti sheep red cell serum Each absorption was performed for 2 hours at room temperature

Absorption of anti D with unsensitized red cells was performed at 37° C for 2 hours by mixing one volume of anti D serum diluted 1 2 and 2 volumes of packed $O_1R_1R_2$ red cells

Reduction by mercaptoethanol Equal volumes of serum and 0 3 M 2 Mercapto

anti D serum used to detect the anti antibodies was tested Equal volumes each of γ globulin or anti D serum exhausted of anti D activity, and of anti antibody serum in *twofold dilutions* were mixed After ½ hour anti D sensitized red cells were added to each tube Readings were performed after 2 hours of incubation No inhibition of anti antibody activity was obtained by native γ globulin while complete inhibition of the anti Gm factor used as control was exhibited (Table 3), even by 1 per mille pooled γ globulin No prozones were observed

The effect of incubating mixtures of incomplete anti D serum, anti-antibody serum and unsensitized red cells was examined Equal volumes of anti D serum and anti antibody serum, both in serial twofold dilutions were mixed The mixtures were left at room temperature for ½ hour 1 per cent unsensitized red cells were added, and the readings performed after 2 hours The typical agglutination pattern of anti antibodies (21) was found The maximum titre of all the anti antibody sera showed that the reaction was similar to that obtained by using sensitized red cells (Table 3)

The anti antibody sera did not agglutinate unsensitized red cells *There was no difference in the agglutination of anti D sensitized red* cells whether unheated or heat inactivated (56° C for ½ hour) sera were used in the tests Each of the 14 sera thus contained antibody activity directed against an antigen antibody complex containing γG globulin and not against native γG globulin

Gm Types

Both Gm and Inv characters are well known markers of heterogeneity of γG globulins Only the Gm test system was available and the factors Gm(a) Gm(x) Gm(b) and Gm(f) were used in the following experiments

The anti antibody sera were Gm typed after mercaptoethanol treatment and after absorption by anti D sensitized red cells The anti D sera were Gm typed after absorption of anti D activity Six anti antibody sera were Gm(a—x—b+f+) (Group I), one was Gm(a+x—b—f—) and one Gm(a+x+b—f—) (Group II) and 6 were Gm(a+x—b+f+) (Group III) (Table 4)

Specificities Demonstrated in Agglutination Reactions

The reactions of anti antibodies with immune complexes containing γG globulin of different Gm specificity were investigated Red cells were sensitized by various anti D sera Anti D 3091 was from a Gm(a+x+b f) donor and specific for Gm(a) and Gm(x) Anti D 3109 and 3083 were from Gm(a—x—b+f+) donors and specific for Gm(b) and Gm(f) respectively (26) Controls included anti Gm(a), anti Gm(b),

rheumatoid factors By exposing serum to preparative ultracentrifugation anti-γG-globulin activity was found in the bottom fractions, further indicating that the active factors were γM-globulins (18)

TABLE 2
Titres of Serum 355 before and after Absorption of the Anti Antibody the Anti Gm Factor and the Heterospecific Rheumatoid Factor Respectively

	Absorption with red cells			
	unsensitized	sensitized by rabbit antibody	sensitized by anti D 3083	sensitized by anti D 3091
Waaler Rose test	512	<8	512	256
Anti D 3083 test	32	16	<2	16
Anti D 3091 test	512	256	256	<4

Controls Unsensitized red cells and serum 355 in dilutions no agglutination
The respective sensitized red cells and saline no agglutination

TABLE 3
Titres of Anti-Antibodies and Anti Gm(f) in Agglutination and Agglutination Inhibition Tests

Anti antibody serum	Red cells sensitized by anti D 3083 Test in			Unsensitized red cells Test in
	saline	1 per cent γ globulin	serum 3083 1 4 absorbed of anti D	anti D serum 3083 1 10
55	64	64	64	64
139	64	32	32	32
141	64	64	64	64
355	32	32	32	32
356	16	16	16	16
576	64	64	32	32
647	32	16	32	32
648	64	64	64	32
649	16	16	16	8
Be	32	32	32	32
Ln	16	16	nd	nd
He	64	32	64	32
Ni	32	16	nd	nd
Sv	32	32	32	nd
Anti Gm(f) A J	64	<2	<2	<2

nd not done
Controls Unsensitized red cells and the respective anti antibody sera in dilutions no agglutination Sensitized red cells and saline no agglutination Sensitized red cells and 1 per cent γ globulin or serum 3083 (1 4) no agglutination

Characterization of Anti-Antibody Activity

A series of experiments was undertaken to characterize the activity of the anti-antibody sera in reaction with human γG-globulin (Table 3)

native γ globulin of the anti D serum employed for the detection of the respective anti antibody activities Furthermore in each case mixing of anti D serum anti antibody serum and unsensitized red cells resulted in strong agglutination Treatment by mercaptoethanol abolished each of the anti antibody activities

It was then investigated whether red cells sensitized by other anti D sera were agglutinated according *to a similar pattern* A panel of 21 anti D sera was selected Anti D 317a and the polyspecific anti CD Ripley were from Gm(a+x+b+f+) donors, 11 Gm(a) anti D antibodies were from Gm(a+b—f—) donors who were Gm(x) positive or negative and 4 Gm(b) monospecific and 4 Gm(f) monospecific anti D antibodies were from *Gm(a—x—b+f+)* donors *The reference anti D* sera (3091 3109 and 3083) were included Some of the sera had previously been tested in absorption experiments (26)

Red cells sensitized by each of the 4 anti D sera specific for Gm(f) only were agglutinated by the anti antibody sera in Group I (55, 139 and 3a) Sera En Ni and Sv were not included for want of serum These anti antibody sera and the anti Gm(f) serum agglutinated red *cells sensitized by the polyspecific anti* CD Ripley On the other hand, red cells sensitized by anti D 317a were agglutinated by the anti antibodies but not by anti Gm(f) These red cells, however, did absorb anti Gm(f) readily indicating that Gm(f) anti D molecules were present on their surface Red cells sensitized by each of the 4 Gm(b) or 11 Gm(a) *monospecific anti D sera were not agglutinated by anti antibodies of* Group I Red cells sensitized by two of the 4 Gm(b) anti D sera (3109 and 3600) were able to absorb anti Gm(f) (26) but they contained much less Gm (f) determinants than anti D 317a (to be published)

Serum 649 from Group II agglutinated red cells sensitized by any of the *Gm(f) anti D antibodies by each of* 9 Gm(a) anti D antibodies (all but 1101 and 1103) by anti D 317a or by anti CD Ripley Red cells *sensitized by* any of the Gm(b) anti D antibodies were not agglutinated Serum Be showed a similar reaction

Three sera (376 647 648) from Group III showed a specificity similar *to Group I anti antibody sera Red cells* sensitized by each of the Gm(f) anti D sera by each of 10 Gm(a) anti D sera (all but 1101) by anti D 317a or by anti CD Ripley were agglutinated by serum 3a6 Red cells sensitized by any of the 4 Gm(b) anti D sera were not agglutinated *Serum He* was not included for want of serum Serum 141 agglutinated *red cells sensitized* by any anti D serum in the panel

Inhibition tests with native γ globulin showed that all these activities detected by the 21 anti D sera were of anti antibody type

The specificity in agglutination reactions and the Gm type of an anti antibody serum were related 1 The anti antibodies from the Gm(a— x b+f+) *donors (Group I)* agglutinated only red cells sensitized by Gm(f) anti D 2 The anti antibodies from the Gm(a+x+b—f—) and Gm (a+x b f—) donors (Group II) agglutinated only red cells sensi

anti-Gm(f) and antiglobulin serum to show the specificity and agglutinability of the sensitized red cells

TABLE 4

Gm Type of Anti-Antibody Sera Titres with Red Cells Sensitized by Various Anti D Sera

	Anti-antibody serum	Red cells sensitized by anti D 3091 Test in saline	anti D 3091 Test in 1 per cent γ globulin	anti D 3109 Test in saline	anti D 3109 Test in 1 per cent γ globulin	anti D 308 Test in saline
Group I	55,139, En,Ni Sv	<2	—	<2	—	16 64
Gm(a−x−b+f+)	355	512	<2	<2	—	32
Group II						
Gm(a+x−b−f−)	649	16	8	<2	—	16
Gm(a+x+b−f−)	Be	32	32	<2	—	32
Group III	141	64	64	8	8	64
Gm(a+x−b+f+)	356	32	32	<2	—	16
	He	64	32	<2	—	64
	576,647,648	<2	—	<2	—	32 64
Controls						
Anti-Gm(a)	3070	512	<2	<2	—	<2
Anti Gm(b)	2535	<2	—	64	<2	<2
Anti Gm(f)	A J	<2	—	<2	-	64
Antiglobulin serum	K 980	512	—	512	—	512

Unsensitized red cells and the respective anti antibody sera in dilutions no agglutination

The respective sensitized red cells and saline no agglutination The respective sensitized red cells and 1 per cent γ globulin no agglutination

The anti-antibody sera gave three types of agglutination patterns (Table 4) In the first type of reaction, represented by sera as 55 and 576, only agglutination of red cells sensitized by the Gm(f) anti D was found In the second type of reaction, represented by sera as He and Be, only agglutination of red cells sensitized by the Gm(a) or by the Gm(f) anti-D was found In the third type of reaction, represented by serum 141, agglutination of red cells sensitized by any of the anti D sera was found However, the reaction was weak using the Gm(b) anti-D Except for serum 355 native γ-globulin did not inhibit the agglutination of red cells sensitized by any of the three reference anti-D sera (Table 4) Agglutination by serum 355 of red cells sensitized by the Gm(a) anti-D was completely inhibited by native γ-globulin as typical for anti-Gm factors, while the agglutination of red cells sensitized by the Gm(f) anti-D was of anti-antibody type

Each of the anti-antibody activities was also characterized in the other tests used above (cfr Table 3) No inhibition was obtained with

native γ globulin of the anti D serum employed for the detection of the respective anti antibody activities Furthermore, in each case mixing of anti D serum, anti antibody serum and unsensitized red cells resulted in strong agglutination Treatment by mercaptoethanol abolished each of the anti antibody activities

It was then investigated whether red cells sensitized by other anti-D sera were agglutinated according to a similar pattern A panel of 21 anti D sera was selected Anti-D 3175 and the polyspecific anti-CD Ripley were from Gm(a+x+b+f+) donors, 11 Gm(a) anti-D antibodies were from Gm(a+b—f—) donors who were Gm(x) positive or negative, and 4 Gm(b) monospecific and 4 Gm(f) monospecific anti-D antibodies were from Gm(a—x—b+f+) donors The reference anti-D sera (3091, 3109 and 3083) were included Some of the sera had previously been tested in absorption experiments (26)

Red cells sensitized by each of the 4 anti D sera specific for Gm(f) only, were agglutinated by the anti-antibody sera in Group I (55, 139 and 355) Sera En, Ni and Sv were not included for want of serum These anti antibody sera and the anti-Gm(f) serum agglutinated red cells sensitized by the polyspecific anti CD Ripley On the other hand, red cells sensitized by anti D 3175 were agglutinated by the anti antibodies but not by anti Gm(f) These red cells, however, did absorb anti-Gm(f) readily indicating that Gm(f) anti-D molecules were present on their surface Red cells sensitized by each of the 4 Gm(b) or 11 Gm(a) monospecific anti D sera were not agglutinated by anti-antibodies of Group I Red cells sensitized by two of the 4 Gm(b) anti-D sera (3109 and 3500) were able to absorb anti-Gm(f) (26), but they contained much less Gm (f) determinants than anti D 3175 (to be published)

Serum 649 from Group II agglutinated red cells sensitized by any of the Gm(f) anti D antibodies, by each of 9 Gm(a) anti-D antibodies (all but 1101 and 1103), by anti D 3175, or by anti-CD Ripley Red cells sensitized by any of the Gm(b) anti-D antibodies were not agglutinated Serum Be showed a similar reaction

Three sera (576, 647, 648) from Group III showed a specificity similar to Group I anti antibody sera Red cells sensitized by each of the Gm(f) anti D sera, by each of 10 Gm(a) anti D sera (all but 1101), by anti D 3175 or by anti CD Ripley were agglutinated by serum 356 Red cells sensitized by any of the 4 Gm(b) anti D sera were not agglutinated Serum He was not included for want of serum Serum 141 agglutinated red cells sensitized by any anti-D serum in the panel

Inhibition tests with native γ-globulin showed that all these activities detected by the 21 anti-D sera were of anti antibody type

The specificity in agglutination reactions and the Gm type of an anti antibody serum were related 1 The anti-antibodies from the Gm(a—x—b+f+) donors (Group I) agglutinated only red cells sensitized by Gm(f) anti D 2 The anti antibodies from the Gm(a+x+b—f—) and Gm (a+x—b—f—) donors (Group II) agglutinated only red cells sensi-

anti-Gm(f) and antiglobulin serum to show the specificity and agglutinability of the sensitized red cells

TABLE 4
Gm Type of Anti-Antibody Sera Titres with Red Cells Sensitized by Various Anti D Sera

	Anti antibody serum	Red cells sensitized by anti D 3091 Test in saline	anti D 3091 Test in 1 per cent γ globulin	anti D 3109 Test in saline	anti D 3109 Test in 1 per cent γ globulin	anti D 3083 Test in saline
Group I	55,139, Fn,Ni,Sv	<2	—	<2	—	16 64
Gm(a—x—b+f+)	355	512	<2	<2	—	32
Group II						
Gm(a+x—b—f—)	649	16	8	<2	—	16
Gm(a+x+b—f—)	Be	32	32	<2	—	32
Group III	141	64	64	8	8	64
Gm(a+x—b+f+)	356	32	32	<2	—	16
	He	64	32	<2	—	64
	576,647,648	<2	—	<2	—	32 64
Controls						
Anti-Gm(a)	3070	512	<2	<2	—	<2
Anti Gm(b)	2535	<2	—	64	<2	<2
Anti Gm(f)	A J	<2	—	<2	—	64
Antiglobulin serum	K 980	512	—	512	—	512

Unsensitized red cells and the respective anti-antibody sera in dilutions no agglutination
The respective sensitized red cells and saline no agglutination The respective sensitized red cells and 1 per cent γ-globulin no agglutination

The anti-antibody sera gave three types of agglutination patterns (Table 4) In the first type of reaction, represented by sera as 55 and 576, only agglutination of red cells sensitized by the Gm(f) anti-D was found In the second type of reaction, represented by sera as He and Be, only agglutination of red cells sensitized by the Gm(a) or by the Gm(f) anti-D was found In the third type of reaction, represented by serum 141, agglutination of red cells sensitized by any of the anti D sera was found However, the reaction was weak using the Gm(b) anti-D Except for serum 355 native γ-globulin did not inhibit the agglutination of red cells sensitized by any of the three reference anti-D sera (Table 4) Agglutination by serum 355 of red cells sensitized by the Gm(a) anti-D was completely inhibited by native γ-globulin as typical for anti-Gm factors, while the agglutination of red cells sensitized by the Gm(f) anti-D was of anti-antibody type

Each of the anti-antibody activities was also characterized in the other tests used above (cfr Table 3) No inhibition was obtained with

native γ globulin of the anti D serum employed for the detection of the respective anti antibody activities Furthermore, in *each case mixing* of anti D serum, anti-antibody serum and unsensitized red cells resulted in strong agglutination Treatment by mercaptoethanol *abolished* each of the anti-antibody activities

It was then investigated whether red cells sensitized by other anti D sera were agglutinated according to a similar pattern A panel of 21 anti D sera was selected Anti-D 3175 and the polyspecific anti-CD Ripley were *from* Gm(a+x+b+f+) donors, 11 Gm(a) anti-D antibodies were from Gm(a+b—f—) donors who were Gm(x) positive or negative, and 4 Gm(b) *monospecific and* 4 Gm(f) monospecific anti-D antibodies were from Gm(a—x—b+f+) donors The reference anti-D sera (3091, 3109 and 3083) were included Some of the sera had previously been tested in absorption experiments (26)

Red cells sensitized by each of the 4 anti-D sera specific for Gm(f) only, were agglutinated by the anti-antibody sera in Group I (55, 139 and 355) Sera En, Ni and Sv were not included for want of serum These anti antibody sera and the anti-Gm(f) serum agglutinated red cells sensitized by the polyspecific anti CD Ripley On the other hand, red cells sensitized by anti-D 3175 were agglutinated by the anti-antibodies but not by anti Gm(f) These red cells, however, did absorb anti-*Gm(f) readily, indicating that Gm(f) anti-D molecules were present on* their surface Red cells sensitized by each of the 4 Gm(b) or 11 Gm(a) monospecific anti-D sera were not agglutinated by anti-antibodies of Group I Red cells sensitized by two of the 4 Gm(b) anti-D sera (3109 and 3500) were able to absorb anti Gm(f) (26), but they contained much less Gm (f) determinants than anti-D 3175 (to be published)

Serum 649 from Group II agglutinated red cells sensitized by any of the Gm(f) anti-D antibodies by each of 9 Gm(a) anti-D antibodies (all but 1101 and 1103), by anti-D 3175, or by anti CD Ripley Red cells sensitized by any of the Gm(b) anti D antibodies were not agglutinated Serum Be showed a similar reaction

Three sera (576, 647, 648) *from Group III showed a specificity similar* to Group I anti antibody sera Red cells sensitized by each of the Gm(f) anti D sera *by each of 10 Gm(a) anti-D sera (all but 1101)*, by anti D 3175 or by anti CD Ripley were agglutinated by serum 356 Red cells *sensitized by* any of the 4 Gm(b) anti-D sera were not agglutinated Serum He was not included for *want of serum Serum* 141 *agglutinated red* cells sensitized by any anti-D serum in the panel

Inhibition tests with native γ-globulin showed that all these activities *detected* by the 21 anti D sera were of anti-antibody type

The specificity in agglutination reactions and the Gm type of an anti-antibody serum were related 1 The anti-antibodies from the Gm(a—

tized by Gm(a) or Gm(f) anti-D 3 Anti-antibodies from 3 of the 6 Gm (a+x—b+f+) donors (Group III) agglutinated only red cells sensitized by Gm(f) anti-D The other 3 anti-antibody sera showed equally strong reactions against red cells sensitized by Gm(a) or Gm(f) anti D One of them (141) also agglutinated red cells sensitized by Gm(b) anti-D

DISCUSSION

Different types of anti-γG-globulin factors show a strictly limited specificity Anti-Gm factors and heterospecific rheumatoid factors can be distinguished by absorption (14), and heterospecific rheumatoid factors with different species specificity can be absorbed individually (24) In 5 of the sera here presented independent absorptions of each of the 2 or 3 types of anti-γG-globulin factors were obtained (Tables 1 and 2) The strict specificity and the independent molecular basis for the anti-antibodies in relation to the anti-Gm factor and the heterospecific rheumatoid factors were thus shown

When serum 355 was tested by *Harboe* in 1959 (designated E H) (11, 15) it contained anti-antibody activity, but anti-Gm activity was not detected In a new sample from 1964 anti-Gm(a) was demonstrated This anti-Gm(a) factor may possibly have been "masked", or has developed between 1959 and 1964, but serum from 1959 was not available for control

The basic criterion for classification of anti-antibodies (Milgrom-Grubb type) is their ability to react with γG-globulin of the same species in antigen-antibody complexes, but not with native γG globulin (23) Each of the 14 sera here presented contained anti-γG globulin factors which were 1 not inhibited by 1 per cent pooled native γ-globulin, 2 not inhibited by native γ-globulin from certain sera although immune complexes containing γG-antibodies from the same sera readily reacted, 3 able to react with human γG-immune complexes even when unsensitized red cells, anti D serum and anti-antibody serum were mixed *Possible reaction with complement or with unsensitized red cells* was excluded The anti-antibody nature of these factors was thus definite, and they were γM-globulins

The distribution of Gm-types in the anti-antibody sera was Gm(a) 57 1 per cent, Gm (x) 7 1 per cent, Gm (b) 85 7 per cent and Gm (f) 85 7 per cent Gm(f) is generally found to be inherited together with Gm(b) in Caucasians (25) The frequencies of the Gm(a) and Gm(b) were close to those described by *Harboe* (12, 15), but the Gm(x) frequency was lower in the present material Whether this bears any general relation to the Gm type of anti-antibody sera needs further research

The present findings were in some respects at variance with the current concept that anti-antibodies react with all types of immune complexes containing γG-antibodies of the same species (7, 16, 23) How-

... et Andresen has subgrouped his anti anti-

antibody properties of his serum factors was not given A human antiglobulin of *Andresen* type was recently described (9)

The 14 sera investigated showed at least three types of different serological specificities of anti antibody nature (cfr Table 4) 1 specificity *directed only against red cells sensitized by Gm(f) anti D*, 2 specificity directed against red cells sensitized by Gm(a) or Gm(f) anti D, 3 specificity directed against red cells sensitized by Gm(a), Gm(b) or Gm(f) anti D

The Gm factors are localized to the heavy chains of the γG globulin with their recently detected subgroups The Gm(a) and Gm(f) characters belong to the We subgroup and Gm(b) to the Vi subgroup (20) The present finding thus indicated that anti antibodies of different serological specificity may be directed at least partly against heterogenic determinants of the heavy chains of γG globulin

Thirteen of the anti antibody sera agglutinated red cells sensitized by each of the Gm(f) anti D sera The 14th serum (356) did not agglutinate red cells sensitized by two Gm(f) anti D sera (3066 3203) Similar discrepancies were found with red cells sensitized by two Gm(a) anti D sera (1101 1103) indicating that other structures than the determinants investigated may be involved in the anti antibody reactions

The Gm(a) and Gm(b) sites both belong to the Fc fragment of the γG globulin molecule (8) while the Fab fragment contains the Gm(f) site (17) The anti antibody reaction may not involve the Gm(f) site of the Gm(f) anti D molecules but other structures of the same molecule possibly in some relation to the Gm(a) or Gm(b) sites It has recently *been shown that human anti antibodies reacted with the Fc* fragment of the γG globulin molecules (16) In the present study red cells sensitized by anti D 3175 from a Gm(a+x+b+f+) donor were agglutinated by the anti antibodies by anti Gm(a) and by anti Gm(x) These red cells were not agglutinated by anti Gm(f) although the anti D antibody 3175 showed a high proportion of Gm (f) molecules demonstrable by absorption experiments (to be published)

Anti antibody specificity and Gm types were further related Red cells sensitized by Gm(a) or Gm(b) anti D were only agglutinated by anti antibodies from Gm(a) and Gm(b) positive donors respectively All the sera agglutinated red cells sensitized by Gm(f) anti D Each of the 14 anti antibody sera thus contained activity directed against red cells sensitized by anti D of at least one Gm type represented in the anti antibody serum Through identifications in the Gm systems anti antibodies seem to be autospecific This is *further demonstrated in Gm(a—x—b+f+)* serum 355 where an isospecific anti Gm(a) factor coexisted with an anti antibody directed against red cells sensitized by Gm(f) anti D

It is well known that only a minor proportion of antibody producing cell clones express Gm(b) while a majority express Gm(a) or Gm(f) (25) On the basis of the above hypothesis it is thus reasonable that only few or a small proportion of anti-antibodies are directed against Gm(b) γG-globulin The finding that only one anti-antibody serum (141) showed specificity for Gm(b) anti-D, and even in a low titre, is in accordance with this view

Most of the anti-antibody sera contained only agglutinating activity against red cells sensitized by anti-D molecules representing some of the Gm types in the respective anti-antibody sera This may indicate that one anti-antibody molecule is only directed against one type of γG globulin, and that polyspecificity in some sera is due to the existence of anti-antibody molecules of different specificity in the same serum *Harboe et al* (16) recently found an anti-antibody serum which was absorbed of agglutinating activity against anti-Rh antibodies leaving the activity against red cells sensitized by anti-AB (γG class) unchanged Observations to be published further indicated the existence of multiple specificities of anti-antibodies in single sera

SUMMARY

1 Anti-antibody activity was demonstrated in 14 sera In addition 5 of these sera contained heterospecific rheumatoid factors and one serum also contained anti-Gm(a) The independent molecular basis for each of these anti-γG-globulin factors was shown

2 The anti-antibodies showed at least 3 types of serological specificities depending on the Gm specificity of the anti D antibodies used for their detection

3 The findings showed a relation between the Gm type of an anti antibody serum and of the anti-D used for detection of the respective anti-antibody Use of the Gm factors as markers indicated a probable autospecificity of the anti-antibodies

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QUANTITATIVE DETERMINATIONS OF INCOMPLETE ANTI-D ANTIBODIES IN REACTION WITH ANTI-Gm FACTORS

By

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The Gm specificity of incomplete anti-D antibodies has usually been determined by the agglutination of anti-D sensitized red cells by known anti-Gm factors (4, 5, 6, 10).

The anti-Gm inhibiting capacity of eluted anti-D antibodies has been tested in some cases (6, 11). However, the differences between inhibiting and non-inhibiting eluates were small. This was in accordance with the finding that γ-globulin concentration and anti-Gm inhibiting capacity of anti-D sera were not significantly altered after absorption of anti-D (2). *Fudenberg et al.* (3) demonstrated that eluted antibodies had only an insignificant inhibiting effect on antiglobulin serum. Thus only limited information was available through the testing of anti-Gm inhibiting capacity of eluted anti-D.

In some anti-D sera, minor proportions of anti-D molecules of certain Gm specificities not demonstrable by these two methods were recently found (11). Red cells sensitized by such anti-D antibodies were not agglutinated by the corresponding anti-Gm factors but were able to absorb them.

This paper presents observations on the agglutination of and absorption by red cells sensitized by varying amounts of anti-D antibodies of different Gm specificity.

MATERIALS AND METHODS

Red cells. O R_1R_2 (CDe/cDE) red cells from a single donor were stored in acid citrate dextrose solution for maximum 5 days before use (11).

Incomplete anti-D sera. The reference anti-D sera specific for Gm(a), Gm(b) and Gm(f) (3091, 3109 and 3083) used previously were selected (11, 12). Two other anti-D sera (1102 and 3607) were included. Anti-D 1102 from a Gm(a+x−b−f−) donor was specific for Gm(a). Anti-D 3091 and 3607 from Gm(a+x+b−f−) donors were specific for Gm(a) and Gm(x). Anti-D 3109 contained a minor amount of Gm(f) molecules only demonstrable by absorption tests (11), but it was the most

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specific Gm(b) anti D available Anti D 3083 was specific for Gm(f) only These anti D sera did not contain anti Gm activity

Anti-Gm sera Anti Gm(a), anti Gm(b) and anti Gm(f) sera obtained as previously described were used (11)

Normal sera Sera from healthy blood donors of different Gm types served as controls

Anti human gammaglobulin serum The rabbit antiserum (K 980) against human whole serum was used as previously (11)

Sensitization of red cells For agglutination experiments with varying amounts of 2 different anti D sera mixtures of the sera (see later) were used to sensitize 0 05 ml of a 10 per cent solution of red cells For absorption experiments and all experiments with anti D 3175 one to 4 volumes of anti D serum or a mixture of 2 anti D sera were diluted 1 8 and mixed with one volume of packed red cells After sensitization at 37° C for 2 hours, the red cells were washed 4 times in large volumes of saline and used in 0 3 per cent suspension on the day they were prepared

Agglutination test Slide technique (see 11) 5 microlitres each of sensitized red cells antiserum and saline were mixed on glass slides (26 × 6 cm) and incubated at room temperature in a humid chamber under gentle agitation for ½ hour The agglutination was graded from +++ to + by microscopic readings under low magnification

The titre of a serum was expressed as the reciprocal of the highest dilution at

EXPERIMENTS AND RESULTS

Agglutination Experiments Using Varying Amounts of Different Anti-D Sera

Serial twofold master dilutions of the 5 anti D sera were prepared Each dilution of the different rows with Gm(b) anti-D serum 3109 was mixed with an equal volume (0 2 ml) of undiluted Gm(a) anti D serum (1102 3091 or 3607) Gm(f) anti D serum 3083 and blood donor serum (control) respectively To each row of tubes containing dilutions of Gm(f) anti D serum 3083, an equal amount of undiluted Gm(a) anti-D serum (1102, 3091 or 3607), Gm(b) anti D serum 3109 and blood donor serum respectively was added To each row of dilutions of the 3 Gm(a) anti D sera (1102, 3091 and 3607), an equal volume of undiluted Gm(b) anti D serum 3109, Gm(f) anti-D serum 3083 and blood donor serum respectively was added. Equal volumes of two anti-D sera were mixed The proportion of each undiluted sera was then given by the proportion of their respective dilutions The initial dilutions before mixing were recorded This did not influence the final results which only depended on given proportions

Each of the mixtures of sera was used to sensitize red cells which were subsequently tested against antiglobulin serum (K 980) and specific anti Gm sera Anti Gm(a) 3071, anti Gm(b) 2535 and anti Gm(f)

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MATERIALS AND METHODS

Red cells O R_1R_2 (CDe cDE) red cells from a single donor were stored in acid citrate dextrose solution for maximum 5 days before use (11)

Incomplete anti D sera The reference anti D sera specific for Gm(a) Gm(b) and Gm(f) (3091, 3109 and 3083) used previously were selected (11 12) Two other anti D sera (1102 and 3607) were included Anti D 1102 from a Gm(a+x b f−) donor was specific for Gm(a) Anti D 3091 and 3607 from Gm(a+x+b f) donors were specific for Gm(a) and Gm(x) Anti D 3109 contained a minor amount of Gm(f) molecules only demonstrable by absorption tests (11), but it was the most

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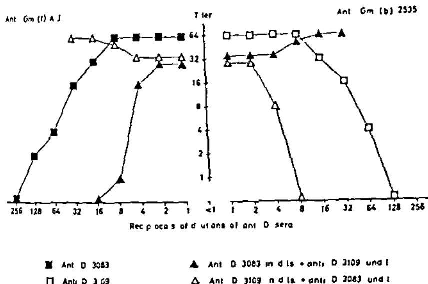


Fig 1

Titre of anti Gm(b) 2535 and anti Gm(f) A.J against red cells sensitized by Gm(b) anti D 3109 or Gm(f) anti D 3083 in serial twofold dilutions (controls) or by mixtures of the two anti D sera. The initial dilutions before mixing of sera are recorded

the reaction soon reached the maximum titre Red cells sensitized by dilutions of Gm(a) anti D serum 3091 mixed with undiluted Gm(b) anti D serum 3109 Gm(f) anti D serum 3083 and blood donor serum respectively showed a reaction very similar to that demonstrated in Fig 1

In these two dimensional tests none of the reference anti D sera (3091 3109 3083) showed a prozone phenomena like that described in one instance by *Masouredis et al* (9) As long as anti D was in excess for sensitization the anti Gm titre was at maximum (giving a horizontal line on the Fig 1) The titre then fell to zero after 8 to 16 fold dilution of the anti D serum The ratio between the highest dilution of anti D serum capable of sensitizing to at least + agglutination with anti Gm serum and the highest dilution giving maximum reaction with anti Gm serum was 1 8 to 1/16

All the batches of sensitized red cells were tested against undiluted anti Gm sera and for control against 2 undiluted blood donor sera The results obtained in some tests with anti Gm(a) are shown in Table 2 In the first row (control) the last + reaction was obtained at the proportion 1 128 (one part of anti D serum 3091 mixed with 128 parts of blood donor serum) When Gm(a) anti D 3091 competed with Gm(b) anti D 3109 or Gm(f) anti D 3083 for sensitization of red cells, the last + reaction was obtained at the proportions 1/16 and 1/8 respectively Similarly the lowest proportions of amount of anti D serum de

A J, containing 64 to 128 agglutinating units of anti-Gm, were used as reference sera Similar results were obtained by anti-Gm(a) 3070 and anti-Gm(b) 2357 When varying amounts of 2 different anti D sera were mixed, anti-D was always added in excess to give maximum sensitization

Each batch of red cells sensitized by the mixtures of anti D sera was strongly agglutinated by antiglobulin serum The titres of the control rows of the anti-D sera in the indirect antiglobulin test were about double those obtained in corresponding tests with specific anti-Gm sera (Table 1)

TABLE 1
Titres of Incomplete Anti-D Antibodies Measured by Antiglobulin Serum (dil 1 32) and Anti Gm Factors (undil)

Anti D serum used to sensitize red cells	Agglutination by			
	antiglobulin serum K980	anti Gm(a) 3071	anti Gm(b) 2535	anti Gm(f) A J
1102	128	64	<1	<1
3091	256	128	<1	<1
3607	256	128	<1	<1
3109	128	<1	64	<1
3083	256	<1	<1	128

Each dilution of anti D serum was mixed with an equal volume of undiluted blood donor serum before sensitization The titres express the reciprocals of the proportions of the two undiluted sera No agglutination was obtained in saline even by undiluted anti D sera

Controls Unsensitized red cells and the respective anti Gm sera (undil) or antiglobulin serum (dil 1 32) no agglutination The respective sensitized red cells and saline no agglutination The respective sensitized red cells and blood donor serum (undil) no agglutination

Sensitized red cells from some rows were tested against twofold dilutions of anti-Gm sera (Fig 1) The ability to react with anti-Gm(b) was lost when the Gm(b) anti D in dilution 1 8 competed with undiluted Gm(f) anti-D for sensitization of red cells In this mixture the proportion of the two undiluted sera used for sensitization was 1/8 (one part to 8 parts) The anti-Gm(b) titre obtained with red cells sensitized by the same dilution, (1 8), of the Gm(b) anti-D in the control row was at maximum On the other hand, when the diluted Gm(f) anti-D had to compete with an increasing excess of Gm(b) anti-D, the reaction with anti-Gm(f) gradually disappeared No reaction was detected when Gm(f) anti-D diluted 1 16 and mixed with undiluted Gm(b) anti-D was used for sensitization The titre obtained using the corresponding dilution 1 16 of the control row was nearly at maximum When equal amounts of the two anti-D sera were mixed undiluted and used for sensitization, the titres were about one half of that obtained by using the same sera individually (Fig 1)

However, using increasing amounts of one type of anti-D molecule,

signated in numerator to anti D serum in denominator at which at least + agglutination was observed, were recorded (Table 3) These proportions differed significantly from those of the respective control rows (cfr Table 1 which shows the titres, here the reciprocals of the respective proportions)

TABLE 3

Anti D sera	Anti-Gm sera	Proportions of anti D sera	Estimated proportions of anti D molecules
Gm(a)3091 Gm(b)3109	Anti Gm(a)	1/16	1/8
Gm(a)3091 Gm(f)3083	Anti Gm(a)	1/8	1/8
Gm(a)3607 Gm(b)3109	Anti Gm(a)	1/16	1/8
Gm(a)3607 Gm(f)3083	Anti Gm(a)	1/8	1/8
Gm(a)1102 Gm(b)3109	Anti Gm(a)	1/8	1/8
Gm(a 1102 Gm(f)3083	Anti Gm(a)	1/8	1/16
Gm(b 3109/Gm(a)3091	Anti Gm(b)	1/1	1/2
Gm(b)3109 Gm(a)3607	Anti Gm(b)	1/1	1/2
Gm(b)3109/Gm(a)1102	Anti Gm(b)	1/4	1/4
Gm(b)3109 Gm(f)3083	Anti Gm(b)	1/4	1/8
Gm(f)3083 Gm(a)3091	Anti Gm(f)	4/1	4/1
Gm(f)3083 Gm(a)3607	Anti Gm(f)	2/1	2/1
Gm(f)3083 Gm(a)1102	Anti Gm(f)	1/1	2/1
Gm(f 3083 Gm(b)3109	Anti Gm(f)	1/8	1/4

The corresponding proportions of the control rows are given by the reciprocal values in Table 1

Absorption Experiments Using Mixtures of Two Anti D Sera

By mixing anti D sera in proportions corresponding to that giving the first negative reaction in the agglutination experiments above, it was possible to prepare sensitized red cells which were not agglutinated by certain anti Gm factors but were able to absorb them (Fig 2) Gm(f) anti D serum 3083 and Gm(b) anti D serum 3109 were mixed and used for sensitization in the proportions 1/16 (mixed coat I) and [illegible] II [illegible] ted by anti- [illegible] Mixed coat [illegible] anti Gm(f) but not by anti Gm(b) Red cells sensitized with each of the anti D sera SV 1957 SV 1965 3109 and 3083 were also prepared, and red cells exposed to a blood donor serum (3092) served for control absorptions All these sera were from Gm(a—x—b+f+) donors Four subsequent absorptions of the anti Gm(b) 2535 and of anti Gm(f) A J were performed Mixed coat I was able to absorb anti Gm(f) in 2 absorptions while it removed anti Gm(b) in one absorption Mixed coat II removed

TABLE 2

Agglutination by Anti Gm(a) 3071 (undil.) of Red Cells Sensitized by Different Anti D Sera in Varying Proportions

Anti D and ctr. sera	Proportions of sera used for sensitization												
	16/1	8/1	4/1	2/1	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
Gm(a)3091/Ctr 3032	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	—
Gm(a)3091/Gm(b)3109	+++	+++	+++	+++	+++	+++	++	++	+	—	—	—	—
Gm(a)3091/Gm(f)3083	+++	+++	+++	+++	+++	+++	++	+	—	—	—	—	—

Controls: Unsensitized red cells and anti Gm(a) 3071 (undil.): no agglutination
Respective sensitized red cells and saline: no agglutination
Respective sensitized red cells and blood donor serum (undil.): no agglutination

Observations on Anti D 3175

Red cells sensitized by anti-D 3175, from a Gm(a+x+b+f+) donor were agglutinated by anti-Gm(a) but not by anti-Gm(b) or anti Gm(f). On the other hand, these red cells were agglutinated by anti-antibody 55 which showed specificity for Gm(f) anti-D (12). In absorption experiments, red cells sensitized by anti-D 3175 removed anti Gm(a), anti Gm(f) and anti-antibody 55, but not anti-Gm(b) Anti-Gm(f) was easily absorbed showing that a relatively high proportion of Gm(f) anti D molecules was present This absorption was much easier than that obtained by using the mixed coats with minor proportions of Gm(f) anti D and it was very similar to the absorption obtained by red cells sensitized by equal amounts of Gm(a) and Gm(f) anti-D

DISCUSSION

In agglutination experiments, very similar results were obtained in 2 rows where red cells sensitized by anti-D sera mixed in inverse proportions were tested against the corresponding anti Gm factors (cfr Fig 1) In absorption experiments (cfr Fig 2), the differences between the results obtained by red cells sensitized by each of the reference anti D sera (3083 and 3109) and by the red cells sensitized by the mixtures of these sera (mixed coats I and II) were also very similar

However, red cells sensitized by some mixtures of Gm(a) and Gm(b) or Gm(f) anti-D showed deviations from these results when tested for agglutination by anti Gm factors In competition with Gm(a) anti D, rather high amounts of Gm(b) and especially Gm(f) anti-D were required to obtain sensitized red cells agglutinable by anti-Gm(b) or anti Gm(f) (cfr Table 3) By contrast, when the same Gm(a) anti-D in dilutions was mixed with undiluted Gm(b) or Gm(f) anti-D for sensitization, the Gm(b) and Gm(f) competed with Gm(a) anti-D resulting in proportions of 1/8 or 1/16 of sera at the last + agglutination with anti Gm(a) (cfr Tables 2 and 3) These proportions corresponded to those obtained from the results presented in Fig 1 This showed that the deviations were not caused by different avidity of the anti D molecules for the antigenic sites on the red cells As furthermore no anti Gm factors were found in the anti-D sera, the deviations seemed to be due [illegible]) molecules on the red [illegible]

[illegible] proportion of different anti D molecules on the red cell surface reflected the proportion of the same molecules in the solution used for sensitization On this basis, and by measuring the amount of anti-D molecules of certain Gm specificities in sensitizing units, the porportion of the various molecules on the red cell surface was estimated One sensitizing unit (in the formula called unit) was defined as the minimum amount of serum which sensitized red cells to give at least + agglutination in a standard test with

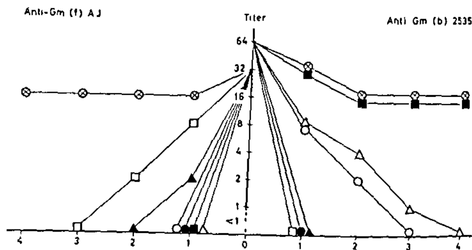


Fig 2

Absorption of anti Gm(b) 2535 and anti-Gm(f) A J by red cells sensitized by mixtures of Gm(b) anti-D 3109 and Gm(f) anti-D 3083 (mixed coats I and II) or by selected control sera The activities of the sensitized red cells in agglutination reaction were tested with undiluted anti Gm sera, — no agglutination +++ strong agglutination After each absorption anti Gm(b) and anti Gm(f) activity was tested with red cells sensitized by reference Gm(b) and Gm(f) anti D respectively

anti-Gm(b) in 4 absorptions and anti-Gm(f) in one absorption The results of the absorptions with red cells sensitized by the other anti D sera were similar to those obtained previously (11, Fig 2)

Absorptions were performed with red cells sensitized by using similar mixtures of each of 2 of the 3 reference anti-D sera (3091, 3109 and 3083) Anti-Gm(b) was removed by another mixed coat (Gm(b) anti-D/Gm(a) anti-D) which was not agglutinated by anti-Gm(b) Anti-Gm(f) was removed by a mixed coat (Gm(f) anti-D/Gm(a) anti-D) which was not agglutinated by anti-Gm(f) Two mixed coats (Gm(a) anti-D Gm(b) anti-D and Gm(a) anti-D/Gm(f) anti-D) absorbed anti-Gm(a) R A and Stang, respectively but were not agglutinated by them However, anti-Gm(a) 3071 was not completely removed in 4 absorptions by these 2 mixed coats It was also more difficult to remove anti-Gm(a) 3071 than anti-Gm(b) and anti-Gm(f) by red cells sensitized only by the respective reference anti-D sera

Observations on Anti D 317a

Red cells sensitized by anti D 317a from a Gm(a+x+b+f+) donor were agglutinated by anti Gm(a) but not by anti Gm(b) or anti Gm(f) On the other hand these red cells were agglutinated by anti antibody aa which showed specificity for Gm(f) anti D (12) In absorption experiments red cells sensitized by anti D 317a removed anti Gm(a) anti Gm(f) and anti antibody aa but not anti Gm(b) Anti Gm(f) was easily absorbed showing that a relatively high proportion of Gm(f) anti D molecules was present This absorption was much easier than that obtained by using the mixed coats with minor proportions of Gm(f) anti D and it was very similar to the absorption obtained by red cells sensitized by equal amounts of Gm(a) and Gm(f) anti D

DISCUSSION

In agglutination experiments very similar results were obtained in 2 rows where red cells sensitized by anti D sera mixed in inverse proportions were tested against the corresponding anti Gm factors (cfr Fig 1) In absorption experiments (cfr Fig 2) the differences between the results obtained by red cells sensitized by each of the reference anti D sera (3083 and 3109) and by the red cells sensitized by the mixtures of these sera (mixed coats I and II) were also very similar

However red cells sensitized by some mixtures of Gm(a) and Gm(b) or Gm(f) anti D showed deviations from these results when tested for agglutination by anti Gm factors In competition with Gm(a) anti D rather high amounts of Gm(b) and especially Gm(f) anti D were required to obtain sensitized red cells agglutinable by anti Gm(b) or anti Gm(f) (cfr Table 3) By contrast when the same Gm(a) anti D in dilutions was mixed with undiluted Gm(b) or Gm(f) anti D for sensitization the Gm(b) and Gm(f) competed with Gm(a) anti D resulting in proportions of 1 8 or 1/16 of sera at the last + agglutination with anti Gm(a) (cfr Tables 2 and 3) These proportions corresponded to those obtained from the results presented in Fig 1 This showed that the deviations were not caused by different avidity of the anti D molecules for the antigenic sites on the red cells As furthermore no anti Gm factors were found in the anti D sera the deviations seemed to be due to interaction between different types of anti D molecules on the red cell surface causing inhibition of agglutination

The present findings thus indicated that the proportion of different anti D molecules on the red cell surface reflected the proportion of the same molecules in the solution used for sensitization On this basis and by measuring the amount of anti D molecules of certain Gm specificities in sensitizing units the porportion of the various molecules on the red cell surface was estimated One sensitizing unit (in the formula called unit) was defined as the minimum amount of serum which sensitized red cells to give at least + agglutination in a standard test with

a given anti-γ-globulin serum (cfr definition of one agglutinating unit) In the present study only anti-Gm factors were referred to A standard amount of undiluted serum then contained a number of sensitizing units given by the respective titre value From the equation

$$\frac{\text{molecules } y}{\text{molecules } z}\ (\text{on red cells}) = \frac{\text{molecules } y}{\text{molecules } z}\ (\text{in solution}) =$$

$$\frac{(\text{units in serum } y)\ (\text{dil of serum } y)}{(\text{units in serum } z)\ (\text{dil of serum } z)} = \frac{(\text{units in serum } y)}{(\text{units in serum } z)} \left(\text{proportion } \frac{\text{serum } y}{\text{serum } z}\right)$$

the proportion of anti-D molecules of various Gm specificities on the red cell surface at the last positive reaction was estimated (Table 3) In the first row in Table 3 (Gm(a) 3091/Gm(b) 3109), serum 3091 contains 128 and serum 3109 64 sensitizing units of Gm(a) anti-D and Gm(b) anti-D respectively The proportion of the respective sera at the last + reaction is 1/16 The estimated proportion of anti-D molecules is then $\left(\frac{128}{64}\right)\left(\frac{1}{16}\right) = \frac{1}{8}$.

The most frequent proportion 1/8 was obtained in 6 instances In 2 instances it was 1/4 and in another 1/16 and these values might be due to methodological deviations The proportions, Gm(b) anti-D 3109 Gm(a) anti-D 3091 and Gm(b) anti-D 3109 Gm(a) anti-D 3607, measured by using anti-Gm(b) were 1/2 The proportions, Gm(f) anti-D 3083 Gm(a) anti-D 3091, Gm(f) anti-D 3083 Gm(a) anti-D 3607 and Gm(f) anti-D 3083/Gm(a) anti-D 1102, measured by using anti-Gm(f) were 2/1 or 4/1 Similar results were obtained after repeated tests Thus the lowest proportion of anti-D molecules of different Gm specificity for detecting the anti-D in deficit in agglutination tests by anti-Gm sera, generally seemed to be about 1/8 This was sustained by the findings in the "two-dimensional tests" with the reference anti-D and anti-Gm sera where a proportion of about 1/8 was found between the highest dilution of anti-D used for sensitization giving at least + agglutination and that giving maximum reaction with anti-Gm serum In certain instances, red cells sensitized by Gm(b) and Gm(f) anti-D were not agglutinated by the corresponding anti-Gm sera although the amount of these anti-D molecules available on the red cell surface exceeded the critical ratio 1/8

The small amount of Gm(f) molecules in anti-D 3109 was not accounted for in the estimations above

The proportion 1/8 giving the limit for agglutination reaction was related to the number of D receptors (probably about 7 8,000 (8)) of the O,R_1R_2 red cell When less than 1 8 of the molecules were of a certain Gm specificity, the number of these molecules per red cell was less than about 1,000 which was thus too little for obtaining agglutination by the anti-Gm factors Different authors (1, 7) found that sensitive antiglobulin sera could just detect from about 100 500 anti-D mole-

cules per red cell In the present study antiglobulin serum w is roughly twice as sensitive as the anti Gm sera (cfr Table 1) This should correspond to a value of about 500 molecules per red cell as the limit for detection of anti D by this antiglobulin serum using the present technique

By mixing suitable amounts of different anti-D sera according to the proportions estimated above, it was possible to sensitize red cells which were not agglutinated by the corresponding anti Gm factor but did absorb it Anti Gm(f), anti Gm(b) and 2 anti Gm(a) sera were absorbed by these sensitized red cells Certain mixtures of anti D sera thus gave experimental models for some sera in this and a previous study (11) These sera contained different proportions of anti D molecules of at least 2 different Gm specificities and one type of molecule was only detected by absorption experiments No significant absorption of anti Gm(a) 3071 was obtained, showing that some anti-Gm factors may be difficult to absorb

In anti D 3175 Gm(a) molecules were demonstrated by agglutination The absorption experiments revealed Gm(f) anti D molecules The findings indicated that anti D 3175 contained Gm(a) and Gm(f) anti D molecules in the proportion of about 1/1 The inability of this anti D to be used for agglutination by anti Gm(f) was probably due to an inhibition similar to that shown when red cells were sensitized by mixtures of Gm(a) anti-D 3091 and Gm(f) anti D 3083 Anti antibody 55 agglutinated and was absorbed by red cells sensitized by anti D 3175 The implication of these findings in the understanding of the specificity of anti antibodies was pointed out in another study (12)

SUMMARY

1 Mixtures containing varying amounts of anti D antibodies of different Gm specificity were used to sensitize red cells The ratio between anti D molecules on the red cell surface reflected the ratio between these molecules in the solution used for sensitization

2 The lowest proportion at which the anti D molecules in deficit on the red cell surface could be detected in agglutination tests was about 1 8 In some instances agglutination was not obtained even when anti D molecules in proportions above this value were used for sensitization

3 Experimental models for some sera containing different proportions of anti D molecules of at least 2 different Gm specificities could be prepared

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PRECIPITATING ANTIBODY RESPONSE TO HERPES SIMPLEX VIRUS IN RABBITS AFTER HERPETIC KERATOCONJUNCTIVITIS

By

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The method of gel precipitation has recently been adapted to studies of herpes simplex virus. *Mala* (1963), with the technique of agar cell culture precipitation, demonstrated two precipitation lines with herpes simplex virus and a rabbit immune serum. *Scott & Tokumaru* (1964), using a herpes simplex antigen prepared from virus grown in rabbit kidney cell culture, found that with some convalescent human sera up to five precipitation lines could be demonstrated and that in density gradient centrifugation the precipitating antigens were distributed among several fractions.

In a previous study at this laboratory (*Mäntyjärvi & Arvilommi* 1964) up to three precipitation lines were obtained with unselected human sera, using concentrated herpes simplex virus antigens prepared from virus grown in HeLa cells. The presence and number of the lines was correlated with the complement fixing and neutralizing antibodies. In the present study a micro gel precipitation method has been used to study the development of precipitating antibodies to herpes simplex virus after experimental herpetic keratoconjunctivitis.

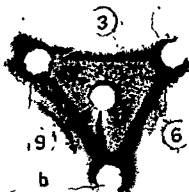
MATERIALS AND METHODS

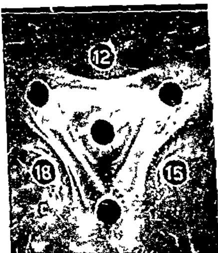
Cell cultures. HeLa cell stock cultures were grown in 1000 ml Roux bottles. The growth medium consisted of 50 per cent Hanks's balanced salt solution (BSS), 40 … The cells were … a 0.02 per cent … ugation and the … and appropriate … tigens, 8 million … For virus titra… … 000 cells in 1 ml … fluent. … was usually con…

The technical assistance of Miss *Pirjo Eino* and Mrs. *Leena Soini* is gratefully acknowledged.

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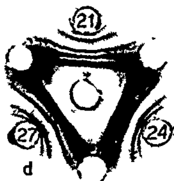






Fig 1

Gel precipitation with subsequent serum specimens from a rabbit with herpetic keratoconjunctivitis Central well control HeLa antigen (Fig 1 a) or herpes simplex antigen (Fig 1 b to 1e) Peripheral wells serum specimens *The number in the well indicates the time in days after virus inoculation*

Preparation of antigen for gel precipitation Virus strain "Kumpulainen", isolated from a patient with recurrent labial herpes infection, was used throughout the study

The method described by *Tokumaru* (1960) for the preparation of concentrated herpes simplex antigens was used with minor modifications HeLa cell monolayers in Roux bottles were washed three times with BSS and 60 ml of maintenance medium consisting of 90 per cent Eagle's minimum essential medium, 5 per cent horse serum and 5 per cent tryptose phosphate broth, was added Bottles were inoculated with 1 ml of herpes simplex virus The dilution of the inoculum was adjusted to give the full cytopathic effect (CPE) in 24 to 36 hours

When the CPE in the bottles was almost complete the cells were mechanically detached, and sedimented by centrifuging for 15 min at 3000 rpm and the maintenance medium discarded The cells were then washed once with saline solution and recentrifuged in a graduated tube The supernatant was discarded, the volume of packed cells recorded and an equal volume of saline or distilled water added The cells were disrupted by freezing and thawing 6 times, the debris removed by centrifuging for 10 min at 2500 rpm, and the supernatant used as antigen in gel precipitation Counted from the original volume of maintenance medium the antigen prepared by this procedure was concentrated about 200 times

Control antigens were prepared identically from uninfected HeLa cells

Virus titration and neutralization HeLa cell culture tubes were washed three times with BSS and 1 ml of maintenance medium was added For titration tenfold dilutions of virus were made in BSS and two tubes were inoculated with 0 1 ml of each virus dilution The tubes were observed daily for CPE and $TCID_{50}$ estimated on the 6th to 7th day

The sera were inactivated by heating at 60° C for 30 min For neutralization equal volumes of virus diluted to contain 100 $TCID_{50}$ per 0 1 ml and twofold dilutions of the sera were mixed The mixtures were incubated for one hour at 37° C and then overnight at 4° C Two HeLa cell tubes were inoculated with each serum virus mixture Serum controls were made by adding to one HeLa cell tube 0 1 ml of serum dilution 1 8 Control titration of virus incubated in the same way as serum virus mixtures was included in each experiment The final reading of the test was performed on the 6th day The serum dilution was considered positive for neutralizing antibodies if not more than a few isolated degenerating plaques were recorded

Complement fixation test The herpes simplex virus antigen for complement fixation was prepared in HeLa cells The micro complement fixation method of *Sever* (1962) was used with minor modifications All the dilutions were made in tubes the serum dilutions with 0 1 ml volumes 0 02 ml was used as unit volume and the reagents dropped onto the plastic panels with syringes and cross cut hypodermic needles calibrated to deliver 1 ml of water as 50 drops The degree of inhibition of haemolysis was visually estimated from 4+ to 0, and 4+ to 3+ was considered positive

Gel precipitation The micro method of *Wadsworth* (1957), as modified by *Raunio* (1964), was used 0 3 per cent agar in distilled water was poured onto a glass slide and allowed to dry Two cover slips were placed on the slide to support a plastic template Ball bearings of steel were put into the holes of the template Melted 1 per cent agar in M/15 phosphate buffer of pH 7 2 was poured between the glass and template After the agar had solidified at 4° C the cover slips were carefully removed and the ball bearings taken off by suction The holes were filled with the

m temperature in a humid atmosphere
oved and non precipitated proteins washed
allowed to dry but the precipitation lines
d (*Uriel* 1964) and photographed in dark

field illumination

Inoculation of rabbits and collection of serum specimens Four adult white rabbits were used The scarified cornea was rubbed with some drops of undiluted herpes simplex virus, strain 'Kumpulainen' grown in HeLa cell cultures The eyes were observed daily Blood samples were obtained before inoculation and thereafter at the intervals indicated The sera were tested for complement fixing, neutralizing and precipitating antibodies All the sera of one rabbit were tested simultaneously

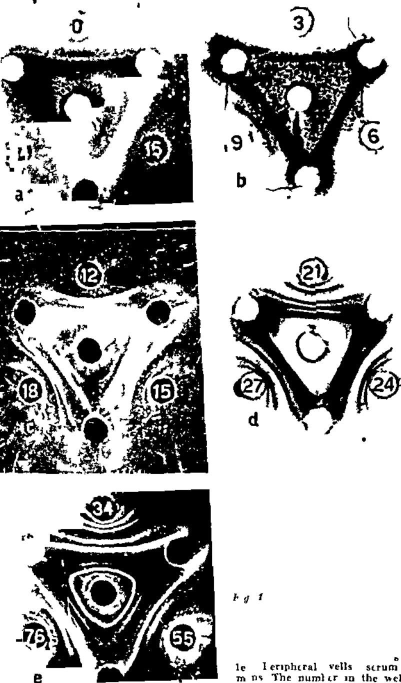


Fig 1

le Leripheral vells scrum speci m ns The numler in the well indi cates the time in days after virus in oculation

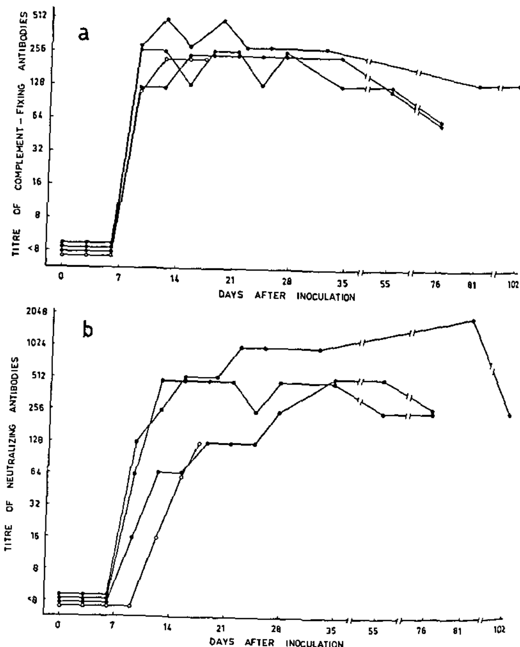


Fig 2

Development of complement fixing (Fig 2 a) and neutralizing (Fig 2 b) antibodies in four rabbits after herpetic keratoconjunctivitis Ocular symptoms appeared on the 3rd day after inoculation One rabbit (open circles) died of encephalitis on the 17th day after inoculation

RESULTS

Typical keratoconjunctivitis developed in each inoculated rabbit The first signs were noticed on the third day after inoculation The conjunctiva became red and the cornea cloudy The eyelids became swollen and at first watery, later a purulent exudate appeared The lesions healed slowly in a week, leaving the cornea more or less cloudy On

the 7th day after the appearance of the ocular manifestations encephalitic symptoms developed in one rabbit, which died a week later, i e on the 17th day after inoculation

In gel precipitation experiments diffuse precipitation was obtained with control HeLa antigens (Fig 1 a) with all serum specimens, including those taken before inoculation With herpes antigens minor differences were obtained between individual rabbits The first one or two precipitation lines were noticed in the serum specimens taken on the 9th or 12th day after inoculation, i e about a week after the ocular symptoms appeared In subsequent specimens the number of lines increased, reaching a maximum of 7 to 8 lines 4 to 5 weeks after inoculation Thereafter the number of lines remained constant throughout the study in one rabbit for three months In Figs 1 b, 1 c, 1 d and 1 e the development of precipitation lines in the specimens of one rabbit is shown

The response of complement fixing antibodies was uniform, as shown in Fig 2 a The titres rose abruptly between the 6th and 9th days after inoculation, stayed at a high level for a month and then began slowly to fall The neutralizing antibody response was similar, although more individual variations were found (Fig 2 b)

DISCUSSION

The diffuse lines of precipitation obtained with control HeLa antigens are not connected with herpes simplex virus, since they were similar in all serum specimens both before and after infection

The number of lines obtained with herpes antigens indicates that at least 8 components were present in the antigen used All these antigen components are apparently closely related to herpes simplex virus, because antibodies to them are developed in rabbits after ocular herpes infection

It will be noticed that the number of precipitation lines continued to increase after the complement fixing and neutralizing antibodies had reached the highest titres

The gel precipitation method, when used with suitable immune sera — combined with electron microscopy, chromatography and differential gradient centrifugation —, should be of value in the study of the antigenic structure of herpes simplex virus It will be interesting to ascertain the time relations in the formation of precipitating and complement fixing antigens and infectious virus particles in the cells It is already known that the soluble complement fixing antigen is formed before the infectious virus (*Gold, Wildy & Watson* 1963) by gel precipitation it might be possible to demonstrate various components in the soluble antigen fraction

SUMMARY

The precipitating antibody response to herpes simplex virus in rabbits after experimental herpetic keratoconjunctivitis was studied by a micro gel precipitation technique Precipitating antibodies were first detected about a week after the appearance of ocular symptoms Two to three weeks later, up to 7 or 8 precipitation lines could be detected, which were all still visible two to three months after infection The first precipitation lines were detected with the same serum specimens in which the complement fixing and neutralizing antibodies were also first demonstrated, but the number of precipitation lines continued to increase after complement-fixing and neutralizing antibodies had reached the maximal level

ADDENDUM

After this paper had been submitted two studies concerning herpes simplex gel precipitation were published by *T Tokumaru* (J Immun 95 181 188 1965 and *ibid* 95 189 195 1965)

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STUDIES ON NON SPECIFIC ANTISTREPTOLYSIN O TITRE

2 *Comparison of Specific and Non Specific Antistreptolysins by Gel Filtration and Dextran Sulphate Precipitation*

By

JOHAN KILLANDER, LENNART PHILIPSON and STEN WINBLAD

Received 18 vi 65

Packalen demonstrated already in 1948 antistreptolysins of unspecific nature bound to lipoproteins (6) Similar observations have been made by subsequent investigators (for a review see ref 10)

In a previous communication it was demonstrated by gel filtration and electrophoresis that γG globulins (for nomenclature see ref 11) carried antistreptolysin activity and that an unspecific antistreptolysin was bound to lipoproteins in the macroglobulin fraction of human serum No antistreptolysin was found to be of γM type (5) Concurrently an efficient procedure for removal of unspecific antistreptolysin has been developed (2) The method is based on the selective precipitation of lipoproteins preferably β lipoproteins from serum with the aid of metal complexes of sulphated polysaccharides (1) Calcium salts of high molecular weight dextran sulphate have been used to deplete human serum of unspecific antistreptolysins This technique is also useful in routine analysis of antistreptolysin (AST) The non specific AST accounts for an average of 40 50 per cent of the numeric value of AST units in sera from blood donors (10) Sera from cases with jaundice with the possible exception of haemolytic jaundice have regularly high titres of non specific AST which are substantially reduced after precipitation of lipoproteins (2 10)

In order to further investigate the chemical nature of the antistreptolysins of specific and unspecific type, sera from cases with jaundice rheumatic fever and a case of monoclonal γG myeloma with very high antistreptolysin activity were studied with gel filtration and dextran sulphate precipitation The results show that dextran sulphate precipitation of lipoproteins in human serum is accompanied by a depletion of the non specific antistreptolysins in the macroglobulin fraction but has no or only a slight effect on the specific antistreptolysins of γG globulin type It is further demonstrated that antistreptolysin activity in the serum from one case of monoclonal γG myeloma is of the γG globulin type and not affected by dextran sulphate precipitation

METHODS

Antistreptolysin (AST) Human sera were tested for antistreptolysin by the colorimetric technique described by *Winblad* (10) The original sera and serum fractions in separation experiments were analysed for antistreptolysin according to *Ipsen* (3) With the latter technique titres were estimated as described by *Packalen & Bergqvist* (7) The titres are expressed in international units (IU)/ml as compared with standard sera from the Department of Biological Standards of the State Serum Institute Copenhagen Denmark

Lipoprotein precipitation by dextran sulphate Lipoproteins were removed by the method of *Burstein & Samaille* (1) To one ml of serum was added 0 02 ml of 10 per cent dextran sulphate 500 (AB Pharmacia Uppsala Sweden) and 0 1 ml of 1 M calcium chloride The mixture was incubated 1 hour at 4° C and subsequently centrifuged at 1500 g for 10 minutes The clear supernate was recovered for antistreptolysin assay and separation by gel filtration on Sephadex G 200

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MATERIAL

To study the chemical type of antistreptolysins in human serum the following cases from the Malmo General Hospital were selected

Case 1

28 year old woman with alcohol addiction complaining of nausea and fever at admission

Signs Enlarged liver sclerae and skin jaundiced

Laboratory findings Bilirubin in serum was between 13 5 54 mg per 100 ml and was also present in the urine Glutamine pyruvic acid transaminase (GPT) 42 units and glutamine oxalacetic acid transaminase (GOT) 320 units Alkaline phosphatase showed a maximum of 320 Buch units

Diagnosis Chronic alcoholic liver cirrhosis

Case 2

54 year old man complaining of nausea pruritus and jaundice at admission

Laboratory findings Bilirubin in serum 38 mg per 100 ml GPT 45 units Alkaline phosphatase 30 Buch units Explorative laparatomy showed a diffuse tumour in the liver Biopsy demonstrated an adenocarcinoma with intense cholestasis

Diagnosis Primary liver cancer

Case 3

40 year old man complaining of acute pain in the joints of the feet and the left knee at admission Anamnestically recurrent tonsillitis and rheumatic fever at 11 years of age The pain later affected the joints of the upper extremities

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(650 cc/m²) by x ray No LL

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Diagnosis Rheumatic fever

Case 4

77 year old man with a classical myelomatosis The serum showed a monoclonal γG globulin This case has been described in detail in a previous communication (9)

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		Before	After	Before	After	Before	After
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2	Primary liver cancer	8500	110	20000	300	2000*	100*
3	Rheumatic fever	1500	850	1250	600	3600*	3200*
4	Monoclonal γG myeloma	175000	100000	215000*	185000*		

The samples marked * were studied by gel filtration

METHODS

Antistreptolysin (ASL) Human sera were tested for antistreptolysin by the colorimetric technique described by *Winblad* (10) The original sera and serum fractions in separation experiments were analysed for antistreptolysin according to *Ipsen* (3) With the latter technique titres were estimated as described by *Packalén & Bergqvist* (7) The titres are expressed in international units (IU)/ml as compared with standard sera from the Department of Biological Standards of the State Serum Institute Copenhagen Denmark

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Diagnosis Primary liver cancer

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40 year old man complaining of acute pain in the joints of the feet and the left knee at admission Anamnestically recurrent tonsillitis and rheumatic fever at 11 years of age The pain later affected the joints of the upper extremities

Signs Sore throat normal heart sounds The joints of the thumb the knuckles and the right foot showed capsular swelling

(650 cc/m²) by x ray No Li
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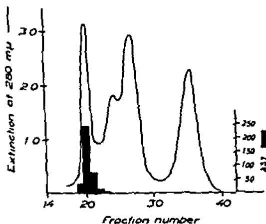


Fig 1

Gel filtration of 3 ml of serum from case 1 (Chronic liver cirrhosis with jaundice) on Sephadex G 200 Protein concentration was determined as optical density at 280 mμ and AST was assayed in each fraction as indicated

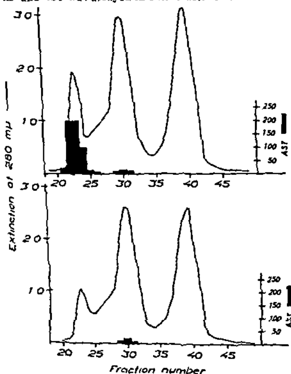


Fig 2

assayed in each fraction as indicated

RESULTS

The Effect of Lipoprotein Depletion on Antistreptolysin Titres

Sera from the cases studied were titrated for antistreptolysin before and after dextran sulphate depletion of lipoproteins Table 1 shows that the selected cases with jaundice had a predominant fraction of non-specific streptolysins precipitated by dextran sulphate but in the cases with rheumatic fever and monoclonal γG myeloma only 14–50 per cent of the total antistreptolysin activity was removed by dextran sulphate treatment These cases therefore appeared suitable for the detection of the chemical difference between the non-specific and specific streptolysins To ascertain that the precipitate formed in the dextran-sulphate procedure was free of γ globulins, the precipitate containing the proposed non-specific antistreptolysins were resuspended after dextran sulphate precipitation and tested for the presence of γ globulins by immunoelectrophoresis The lipoproteins from three additional sera with varying contents of non-specific antistreptolysins were precipitated by dextran sulphate and the precipitate washed 5 times with saline containing 1 per cent dextran sulphate and 0 1 M $CaCl_2$ The precipitate was then resuspended in saline and tested for antistreptolysin activity and examined by immunoelectrophoresis Table 2 shows that the *non-specific streptolysins in the precipitate are quantitatively* recovered after precipitation No γ globulins could be detected in the resuspended precipitates These findings indicate that the dextran sulphate procedure only removes non-specific antistreptolysins

TABLE 2
Recovery of Antistreptolysin and γ Globulins in the Dextran Sulphate Precipitate

Serum No	Antistreptolysin activity in: *Untreated serum*	Supernate after $DxSO_4$ precipitation	Resuspended and washed precipitate after $DxSO_4$ *precipitation*	γ globulin by immune electrophoresis
1	1100	450	800	—
2	5000	125	5600	—
3	800	140	950	—

Gel Filtration of Lipoprotein Depleted and Untreated Sera

It was earlier established that antistreptolysins of antibody type eluted in the γG globulin fraction *after gel filtration on* Sephadex G-200 and that the non-specific antistreptolysin was bound to *lipoproteins in* the macroglobulin immunoglobulins (5) The sera from the described cases were separated by gel filtration on Sephadex *G-200 before* and

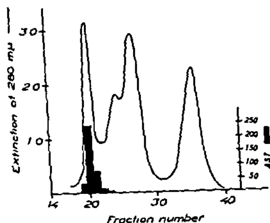


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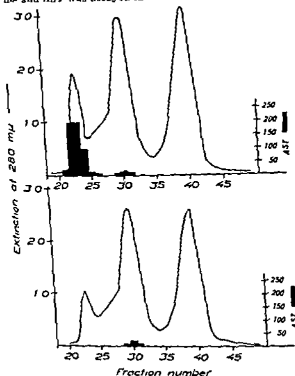


Fig 2

Gel filtration of serum from case 2 (Primary liver cancer with jaundice) on Sephadex B-200 Upper figure gives elution diagram of 3 ml of serum before and lower figure after dextran sulphate precipitation of the lipoproteins from 3 ml of serum Protein concentration was determined as optical density at 280 mμ and AST was assayed in each fraction as indicated

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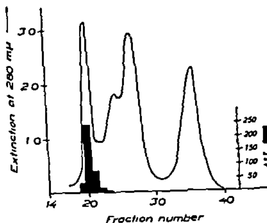


Fig 1

jaundice)
density at

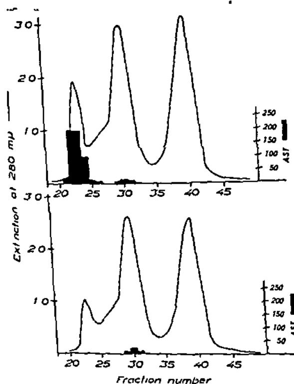


Fig 2

Gel filtration of serum from case 2 (Primary liver cancer with jaundice) on Sephadex B-200. Upper figure gives elution diagram of 3 ml of serum before and lower figure after dextran sulphate precipitation of the lipoproteins from 3 ml of serum. Protein concentration was determined as optical density at 280 mμ and AST was assayed in each fraction as indicated.

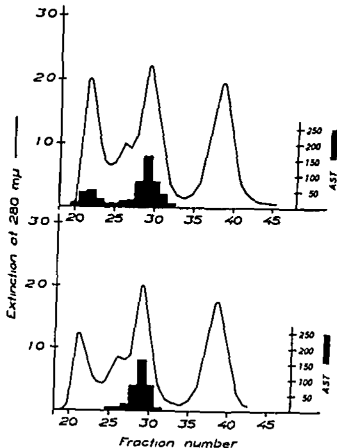


Fig 3

Gel filtration of serum from case 3 (Rheumatic fever) on Sephadex G 200 Upper figure gives elution diagram of 3 ml of serum before and lower figure after dextran sulphate precipitation of the lipoproteins from 3 ml of serum Protein concentration was determined as optical density at 280 mμ and AST was assayed in each fraction as indicated

after depletion of lipoproteins by the dextran sulphate method The activity recovered in the macroglobulin fraction was consequently considered to be of non-specific type and the activity in the γG globulin region of antibody type

Fig 1 shows that the antistreptolysins from case 1 elute in the macroglobulin fraction before dextran sulphate precipitation of lipoproteins After depletion of the lipoproteins antistreptolysin activity was not detected in the fractions obtained by Sephadex G-200 gel filtration Fig 2 shows that the antistreptolysin in the macroglobulin peak was abundant in the serum of case 2 before dextran sulphate precipitation of the sera. After lipoprotein depletion activity is confined to the γG globulin region in the second protein peak An additional case with a diagnosis of infectious hepatitis was also investigated and showed a similar distribution of the antistreptolysins, but the elution diagram has not been included Thus in these cases with jaundice it has been demonstrated that the antistreptolysins are of non-specific type and

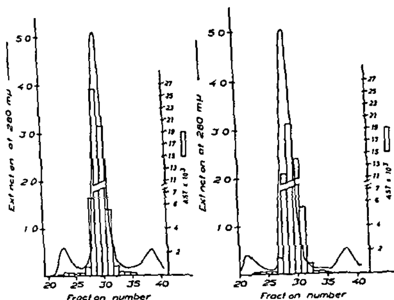


Fig 4
Gel filtration of serum from case 4 (Classical myelomatosis) on Sephadex G 200 To the left the elution diagram of 1 ml of serum before to the right of 1 ml of serum after precipitation of lipoproteins with dextran sulphate Protein concentration was determined as optical density at 280 mμ and AST was assayed in each fraction as indicated

eluting in the macroglobulin fraction probably associated with lipo proteins

The elution diagram from Sephadex G 200 of the sera from case 3 is shown in Fig 3 The untreated sera contain antistreptolysin activity both in the macroglobulin fraction and in the second protein peak After removal of lipoproteins from the serum by dextran sulphate the activity is confined to the γG globulin region in the second protein peak

It has earlier been established that the high antistreptolysin activity associated with cases of monoclonal γG myeloma is not affected by dextran sulphate precipitation (9) In accordance the main part of the antistreptolysins from case 4 eluted in the γG globulin region before and after precipitation of the lipoproteins by dextran sulphate as shown in Fig 4 A small amount of antistreptolysin in the macroglobulin peak before dextran sulphate precipitation was not recovered after such treatment

The recovery of non specific and specific antistreptolysins after gel filtration on Sephadex G 200 is complied in Table 3 The recoveries range from 62 75 per cent of non specific antistreptolysin and from 38–98 per cent of the specific antibody Since the serum volumes were determined prior to dextran sulphate precipitation and small losses occurred when the supernatant was transferred to gel filtration, a re-

TABLE 3

Recovery of Antistreptolysin Activity by Gel Filtration on Sephadex G 200

Case No	Elution diagram in Figure No	Recovery in per cent of applied activity				Ratio AST before and after lipoprotein depletion	Ratio AST $\frac{M + \gamma G}{\gamma G}$*
		Macroglobulin fraction Lipoprotein depletion		γG globulin region Lipoprotein depletion			
		None	After	None	After		
1	1	72	0	0	0	17	20
2	2	65	0	4 5	90	20	14
3	3	27	0	34	38	1 1	1 7
4	4	0 7	0	81	98	1 7	1 1

* M denotes total AST in macroglobulin fraction and γG denotes total AST in the γG region

duced recovery would be anticipated Thus both the gel filtration and the dextran sulphate methods appear to efficiently separate specific from non specific antistreptolysin

DISCUSSION

The separation of non specific and specific antistreptolysin is achieved by gel filtration on Sephadex G 200 since the non specific activity elutes together with the macroglobulins probably associated with lipoproteins and the specific antibody eluted in the γG globulin region in the second protein peak (5) In the present investigation this was confirmed by investigating the elution diagram of serum depleted of lipoproteins by the dextran sulphate procedure described previously (10) Since no antistreptolysins have been found to be of γM globulin type (5) it appears that all activity recovered in the macroglobulin fractions is of the non-specific type

In confirmation of previous results obtained with the dextran sulphate procedure, which eliminates the non specific antistreptolysins, cases with jaundice show predominantly the non specific type of antistreptolysins in gel filtration experiment

The antistreptolysin from a case of monoclonal γG myeloma is not affected by dextran sulphate treatment nor is it eluted in the macroglobulin fraction indicating by the criteria mentioned above that this activity is of the specific type Purified γG globulin from case 4, however showed no precipitation in agar gel against purified streptolysin O in contrast to γG antibodies from a case of rheumatic fever (unpublished) Thus this activity in the γG globulin region must therefore be further investigated before its specific character can be established

The results with the gel filtration technique and the analysis of the dextran sulphate precipitate indicated that the dextran sulphate procedure did not coprecipitate any significant amounts of specific anti streptolysin activity

The non specific character of the lipoprotein bound antistreptolysins has recently challenged *Rowen* (8) *suggest that mice are refractory to* the lethal effect of streptolysin O on subsequent challenge because of a lipoprotein in plasma with antistreptolysin activity

SUMMARY

Gel filtration on Sephadex G 200 of human sera containing antistreptolysin showed that non *specific activity associated with lipoproteins* eluted in the macroglobulin fraction and was separated from the specific activity in the γG globulin region The dextran sulphate procedure to precipitate lipoproteins appears to eliminate the non-specific activity but does not affect the specific antibody

Cases with jaundice have a predominant fraction of non-specific antistreptolysins and a single case of monoclonal γG myeloma appears to have specific antistreptolysins

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Acta path et microbiol scandinav 65, 597 599, 1965

The Enterovirus Department Statens Seruminstitut, Copenhagen Denmark

GROWTH OF RUBELLA VIRUS IN A RABBIT KIDNEY CELL LINE RK 13

By

J Leerhoy

Received 26 vi 65

The propagation of rubella virus in tissue culture has been studied by *Parkman et al* (1) and *Plotkin et al* (2) in cell systems where the activity of the virus was recognized by interference with the growth of echo 11 virus This report describes a rubella virus growth curve study in a rabbit kidney cell line, RK 13, in which rubella virus has been found to produce very characteristic microfoci in the cell sheet (3) The virus activity was measured by observation of this direct cytopathic effect The experiment was performed in order to determine the time for maximum virus yield and also the relationship between free and cell associated rubella virus

MATERIALS AND METHODS

Virus Rubella virus strain Judith kindly supplied by Dr *A Svedmyr* Stockholm was used in its fourth RK 13 tissue culture passage in this laboratory The titre was approximately 4 2 log 10 TCID 50 per ml

Titrations Serial tenfold dilutions of the virus prepared in medium 199 with the same additions as the maintenance medium were inoculated in 0 2 ml aliquots on five tube cultures each containing 1 8 ml of maintenance medium The tubes were rotated at 34° C and examined on the third and fourth day after inoculation Appearance of microfoci in the cultures was interpreted as presence of rubella virus and fifty per cent infectivity end points were calculated by the method of Karber All titres are given per ml of virus

EXPERIMENTS AND RESULTS

Growth curve experiment RK 13 monolayer tubes 3–5 days old were changed to maintenance medium and inoculated with approximately

This study was part of work undertaken while the author held a scholarship grant from the *Royal Veterinary and Agricultural College Copenhagen*

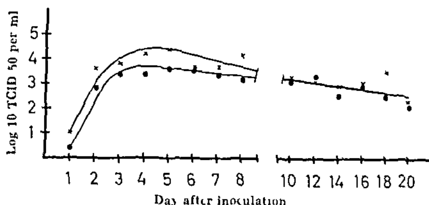


Fig 1
Growth of rubella virus in RK 13 cells at 34° C
● Free virus × Cell associated virus

100 TCID 50 of rubella virus The tubes were rotated at 34° C At intervals of 24 hours eight tubes were harvested for sampling of free and cell-associated virus, the first samples being collected 24 hours after inoculation of the tubes The medium from four of the eight tubes was pooled without further treatment, the harvests representing free virus The remaining four tubes were subjected to three cycles of freezing and thawing before the contents were pooled This material will be referred to as cell-associated virus The pools were centrifuged at 1500 r p m for 10 minutes and stored at —60° C until all samples were collected and simultaneous titrations could be performed

Results The results of the growth curve experiments are recorded in Fig 1 As can be seen the medium contained only little free virus for the first 24 hours after inoculation During the next 24 hours the concentration of free virus increased rapidly from 0.4 to 2.8 logs and a maximal titre of about $10^{3.6}$ was reached five days after inoculation On continued incubation the titre of free virus decreased slowly throughout the remaining observation period reaching values of about $10^{2.2}$ on day 20

The curve representing the titres of cell-associated virus followed a course almost parallel to the curve for free virus The curve reached its peak of 4.4 logs on day 5, when a slow and continuous decrease began The lowest value, 2.4 logs, was seen at the end of the observation period, *i e* on day 20

DISCUSSION

The growth curves for free and cell-associated rubella virus in RK 13 cells both reach a maximum at approximately day 5 Thereafter the virus titres decrease rather slowly, approximately 2 logs during the 20-day-observation period During the period of rapid virus multiplication, *i e* day 1 through day 5, the cell-associated virus titres (virus in disintegrated cells suspended in the virus containing medium) are 0.6 to

0 8 log higher than the titres of free virus This indicates that most of the virus particles are associated with the tissue culture cells For preparation of potent antigens, disintegrated cells suspended in a small volume of medium might therefore be advantageous

When rubella virus is kept in maintenance medium without cells at 37° C, the rate of inactivation has been found to be of an order of 0 3 to 0 4 log per hour (1) The very slow decrease in titre observed in the present experiments after day 5 would seem to disagree with this finding The gradual, slow decrease in titre from day 5 to day 20 in both growth curves would therefore seem to reflect a balance between newly formed infective particles and heat inactivation

SUMMARY

The growth of rubella virus has been studied in a rabbit kidney cell line RK 13 Maximal infectivity titres were obtained approximately 5 days after inoculation From this time the virus titres decreased slowly for the rest of the observation period The titre of cell associated virus was during the first week 0 6 to 0 8 log higher than the titre of the cell free culture medium, indicating that most of the virus particles were associated with the tissue culture cells

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Acta path et microbiol scandinav 65, 600–606, 1965

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STUDIES ON ANTIGEN PREPARATIONS FROM *STAPHYLOCOCCUS AUREUS*

1. *The Influence of Modification of Functional Groups and Enzymic Digestion on the Serological Activity of Protein A*

By

ARNE GROV

Received 22 vi 65

In two previous papers (4, 11) the isolation, purification, and chemical and serological characterization of *protein A* (antigen A) have been described Besides the precipitinogen giving a specific line with normal human sera on agar gel precipitation, and a double line with rabbit immune serum against the homologous bacteria, the preparation was shown to contain an antigen capable of sensitizing tanned sheep erythrocytes for the haemagglutination test The antigens were shown to be of protein nature Although the purified preparation apparently possesses three different antigens, no separation was obtained on electrophoresis However, a column fraction containing only sensitizing substance was obtained (4) The stability of the serological activity of the antigens, when exposed to the rather rough conditions during preparation, may indicate that the molecules are rather simple polypeptide chains

The present study deals with the effects of specific modifications of the functional groups and of enzymic digestion on the biological activities of protein A The functional groups probably play a prominent rôle in the biological interactions in question, and the purpose of the investigation was to gather information about the biological activity of the various groups

MATERIAL AND METHODS

Antigen preparation The sample of protein A used in this study was prepared according to the procedure described in (4)

Reagents 2,4 dinitro 1 fluorol
phenyl *iso* thiocyanate (PITC) fr
from nitrosomethylurea (Fluka)
(1) Carboxypeptidase-A, a toluene H_2O suspension was obtained from Sigma Chemical Company, and crystalline trypsin from Novo Copenhagen

Modifica

Blocks
by *Mills*
bicarbona
containing 0 025 ml of DNFB, the sample was shaken at room
dark, for 5 hrs. The solution was then diluted with 4 ml of water and excess DNFB extracted with two portions of 2 ml of peroxide free ether The ether-extracted sample was evaporated to dryness *in vacuo* over KOH pellets and then taken up in a measured volume of water

Modification of the amino groups by PITC
(13) using from 0 2–2 mg of protein A and 0 1
reagent and the pyridine by three extraction
aqueous layer was evaporated to dryness *in vacuo*,
up in a measured volume of water Day light was excluded as far as possible

Modification of acid groups by diazomethane was performed by dissolving the antigen in a minimum quantity of water and then adding it to the ether containing diazomethane After shaking for one hour at room temperature, the mixture was evaporated to dryness *in vacuo* and then taken up in a measured volume of water

Determination of Terminal Amino Acids

means of both the DNFB

extracts and the extracted hydrolysate were evaporated to dryness *in vacuo* over KOH pellets *The residues were subjected to circular paper chromatography* As references, authentic samples of DNP amino acids obtained from Nutritional Biochemicals Co (Cleveland Ohio) were used During preparation and subsequent

concentrated and analysed by circular paper chromatography Standard PTH amino acids were prepared according to the procedure described in (13)

Examination of C terminal amino acids was performed by means of carboxypeptidase (5) One mg of protein A and 0 05 mg of carboxypeptidase A were dissolved in 1 ml of a 0 1 per cent solution of sodium bicarbonate The pH of the solution was ad to 8 5 and the mixture was incubated at 37° C At different times
g of Amberlite IR 120
tubes were shaken for
n was then thoroughly
ml aq 5 N ammonia
solution for 20 mins each Both the water and the ammonia extracts were dried in vacuo over conc sulphuric acid and examined for free amino acids by circular paper chromatography

Solvent Systems for Chromatography

100–120°

Spray Reagents

a) Iodine-azide starch reagent (13) for the detection of PTH amino acids
b) Ninhydrin, 0.5 per cent in acetone, for the detection of amino acids (3)
c) Isatin for the detection of proline (8)

Whatman No. 1 filter paper was used throughout this investigation and the chromatographic technique used was that described in (3).

Reactions with trypsin were carried out at pH 7.4 (0.2 M phosphate buffer) and 37° C (0.1 per cent soln., enzyme/substrate ratio, 1/100 by wt.) Varying incubation times were used.

Serological Methods

Immune serum against the homologous strain Cowan I was produced as described by *Oeding* (10). The *human serum* used in this experiment was a pool of 10 normal human sera.

The *agar gel precipitation* was carried out according to the method used by *Haukenes & Oeding* (6).

The *haemagglutination test* was performed essentially as described by *Morse* (9) using a 1 : 40 000 solution of tannic acid for tanning the sheep erythrocytes.

RESULTS

All the modification reagents changed the serological activities. The agar gel precipitation test showed at first that by modifying the amino groups by DNFB and PITC as well as the carboxyl groups by diazomethane, the precipitation line with human serum and the corresponding line with Cowan I antiserum disappeared while the specific Cowan I line was still present, and visually unaffected. When larger amounts of the reagents were used, both precipitation lines disappeared, showing that the first reactions had not been quantitative. No precipitation line could be observed on further concentration of the reaction products.

Two hours' incubation with trypsin led to destruction both of the sensitizing and precipitating ability of protein A, whereas the specific precipitation line with Cowan I antiserum was still present after incubation for 30 minutes.

When protein A was digested with carboxypeptidase, no decrease in the activities could be observed on agar gel precipitation. The precipitation line with human serum and the double line with Cowan I antiserum were still present after digestion for 6 days using different quantities of enzyme. After shaking the solution with ion exchange resin (Amberlite IR 120), the supernatant gave a positive reaction on agar gel precipitation while the aq. NH_3-extract, after having been evaporated to dryness and dissolved in water, gave a negative reaction.

In the haemagglutination test, 0.2 mg samples of the antigen were used. The residues, after evaporation to dryness of the reaction products with the various modification reagents, were taken up in 2 ml of saline. The reaction products with PITC were dialysed for 48 hrs against a continuous flow of tap water before evaporation *in vacuo*. The pH of the enzyme digestions was adjusted to 7 before sensitization. The haemagglutinating ability was completely removed by all treatments. Samples of protein A carried through the same operations without

addition of modification reagents gave the same haemagglutinating titres as an untreated sample

Chromatographic examination for DNP-derivatives in solvent systems A, B, and E revealed the presence of DNP-alanine and a trace of ε-DNP-lysine

The acetone extracts of the reaction products of protein A with PITC were subjected to chromatography in solvent systems C and D Spray *reagent* a) revealed *one* band with movement identical to that of standard PTH alanine

Chromatography of the supernatant and ammonia extract from the carboxypeptidase experiments was performed with solvent systems E and F using spray reagents b) and c) No free amino acids could be observed in the supernatant *In the aq* NH_3*-extracts*, the same amino acids as in hydrolysed protein A, except proline, were observed after 2 hrs incubation lysine, aspartic acid, glutamic acid, glycine, serine, alanine, valine, and leucine An aq NH_3 extract after half an hour's incubation showed only aspartic acid and a trace of glycine The 6 N hydrochloric acid hydrolysate of the supernatant after complete digestion with carboxypeptidase, contained all the amino acids found in undigested protein A, as demonstrated chromatographically

DISCUSSION

Absorption experiments have previously shown that the sensitizing ability of protein A is due to a distinct antigen different from the precipitinogen (11) The present modification experiments indicate that the two precipitation lines observed with Cowan I antiserum are due to two separate precipitinogens This is supported by absorption When a solution of protein A was absorbed with human serum, only the specific Cowan I line was observed on agar gel diffusion using Cowan I antiserum

Alteration in a functional group of a protein may produce a change in a serological activity in either of two ways by altering the configuration of the molecule or by removing a group generating the property It is not possible to establish whether the disappearance of serological activities observed after the various modifications is due to an alteration of the active sites only, without interference from secondary modifying effects *on the rest of the molecules By blocking func*tional groups the distribution of the electrons in the molecule is changed thus altering or preventing the electrostatic interaction between antigen and antibody This may explain why the same result is obtained by modifying both amino and carboxyl groups of protein A The reaction with DNFB, PITC, and diazomethane did not seem to produce any specific effects, and it seems likely that amino as well as carboxyl groups of protein A are engaged in the activities

The most obvious difference with respect to the two precipitation

lines of protein A is revealed by the experiments with different incubation times on tryptic digestion The line with human serum and the corresponding line with Cowan I antiserum always disappeared first, and this fact did not seem to be related to the relative quantities of the precipitinogens, since both give approximately the same activities in untreated samples

Both precipitation lines disappeared with all modification methods tested when larger amounts of reagents were used Since amino or carboxyl groups are involved in all instances, it is likely that the groups responsible for the specific Cowan I line are less accessible or susceptible to modification Possibly, they are more sterically shielded or chemically less reactive than the groups which are responsible for the precipitation line with human serum

Neither of the two antigens giving rise to the two precipitation lines were destroyed by digestion with carboxypeptidase, and no decrease of their precipitation activities could be observed This shows either that the precipitating molecules have no free C-terminal amino acid or that attack by the enzyme is sterically hindered The enzyme fails to release amino acids directly attached to proline (5) Chromatographic examination of the ammonia extract showed that except for proline, all amino acids found by acid hydrolysis of protein A were set free by the enzyme Most of the released amino acids are certainly derived from the destroyed sensitizing substance, but as demonstrated earlier (4), the amino acids valine and leucine are not engaged in the sensitizing ability Most likely, therefore, these acids are C-terminals of the precipitinogens, and if so, they are not essential for the serological specificity Acid hydrolysis of material digested with carboxypeptidase showed, however, that valine and leucine also contribute to the composition of the rest of the molecules with all the other amino acids found in the antigenic material

As the ability to sensitize tanned sheep cells is destroyed by carboxypeptidase digestion, it must be due to an open polypeptide chain with free C-terminal amino acid Carboxypeptidase applied to the problem of identifying C-terminal residues of protein A was not entirely successful, but the findings after incubation for half an hour may lead to the conclusion that aspartic acid is a C-terminal amino acid Aspartic acid was the only free amino acid found on weak acid hydrolysis of protein A (4), another indication that it is in a terminal position However, it cannot be said with certainty whether the released aspartic acid is part of the antigen sensitizing tanned sheep cells or whether it is associated with one or both of the precipitinogens Since aspartic acid was found in trace amounts in the column fraction sensitizing tanned cells (4), it possibly contributes to this activity

Only PTH-alanine was released by the PITC treatment, and DNP-alanine together with some ε-DNP-lysine by the DNFB treatment This shows that alanine is the only N-terminal amino acid Both sensitizing

and precipitating substances may thus have N-terminal alanine, and most likely the latter as PTH alanine is also released after carboxypeptidase treatment

Since only a trace of ε DNP lysine could be observed, it is suggested that lysine carries a single alanine substitute attached to the ε-NH_2 group or a peptide chain with *N-terminal alanine*

It seems likely that our protein A preparation is composed of three different molecular entities which are intimately interrelated, one being capable of sensitizing tanned sheep cells, and two giving rise to two different precipitation lines Their different antigenic specificities seem *to be due to variations in their amino acid* sequences and their spatial arrangements

SUMMARY

The effect on the serological activities of protein A of chemical modifications of the functional groups carried out by 2,4 dinitro-1-fluorobenzene (DNFB), phenyl *iso*-thiocyanate (PITC), and by diazomethane have been studied Agar gel precipitation and haemagglutination tests with the reaction products as antigens, showed complete loss of serological activity by blocking either of the amino or of the carboxyl groups

Modification and absorption experiments indicate the presence of three different molecular species, one with sensitizing ability and two acting as precipitinogens

By the DNFB and the PITC method it could be shown that alanine is the only N terminal amino acid Most probably it is a constituent of the precipitinogens

Digestion of protein A with carboxypeptidase-A destroyed the haemagglutinating ability, while the precipitating activities remained unchanged This indicates that the haemagglutinating ability is due to an open peptide chain with free C terminal, and that the action of the enzyme on the precipitating substances is most probably blocked by proline

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STUDIES ON ANTIGEN PREPARATIONS FROM *STAPHYLOCOCCUS AUREUS*

2 *The Influence of Modification of Functional Groups and Enzymic Digestion on the Serological Activity of Polysaccharide A*

By

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Received 23 vi 65

Polysaccharide A of *Staphylococcus aureus* has been found to be a polymer of 4-0-β-N acetylglucosaminyl-ribitol phosphate (or ribitol teichoic acid) associated with a mucopeptide moiety (8) The specificity of the precipitation line on agar gel diffusion produced by polysaccharide A and homologous immune serum is due to the β-linkage of N acetylglucosamine (10) In addition to its precipitating ability, polysaccharide A also sensitizes tanned sheep cells to haemagglutination The chemical basis of the sensitizing ability has not been established It has been shown that pure teichoic acid is incapable of sensitizing tanned sheep cells (12) By column fractionation of crude polysaccharide A, *Oeding et al* (12) were able to isolate a fraction which sensitized tanned sheep cells but did not give precipitation in the agar gel test The isolated substance contained the same amino acids as did the mucopeptide moiety of polysaccharide A A similar protein substance isolated from crude protein A (extract A) (6) showed serological property closely related to that of the substance isolated from polysaccharide A

The present paper deals with the effects of some modification experiments and of digestion with different enzymes on the serological activities of polysaccharide A

MATERIALS AND METHODS

The *polysaccharide A preparation* used in this study was prepared as described by *Haukenes* (7) from strain Wood 46 The final fractionation on Dowex-1 column was omitted

The *chemical modification procedures the tryptic and carboxypeptidase digestions* and the methods for *chromatographic examinations* were the same as those described in (5)

Digestion with β N acetylglucosaminidase was performed according to the description of *Findlay & Levvy* (1) The enzyme preparation used was kindly furnished by Professor *Levvy*

Reactions with acid phosphatase (from wheat germ, Type 1, Sigma Chemical Company) were carried out at pH 4 8 (0 1 M citrate buffer) and 37° C (0 1 per cent soln, enzyme/substrate ratio, 1/100 by wt) Varying incubation times were used

Periodate oxidation was performed in 0 1 per cent sodium periodate solution One mg of polysaccharide A per ml of solution was heated to 37° C for 2 hrs and was then dialysed for 24 hrs against running tap water

Mild alkaline hydrolysis was carried out in N ammonium hydroxide (0 1 per cent solution) for 5 minutes at 100° C, after which the solution was cooled immediately The ammonia was removed by evaporation under reduced pressure (9)

A column of ion exchange resin, Dowex 50 W_{X4} 20–50 mesh (wet), was used to isolate the active materials after enzymic digestions Control samples were included in all experiments in which alterations in serological activities were studied

RESULTS

Of the modification methods used, only the diazomethane treatment destroyed the precipitating ability of our polysaccharide A preparation Both phenyl-*iso*-thiocyanate (PITC), 2,4-dinitro-1-fluorobenzene (DNFB), and diazomethane completely destroyed the sensitizing ability Even small amounts of these reagents reduced the haemagglutination titre, while in controls without reagents, the titre remained unchanged.

Digestion with β-N-acetylglucosaminidase destroyed the precipitating ability The haemagglutination titre was strongly reduced, but the same reduction was observed in a control sample without the enzyme

Acid phosphatase released phosphoric acid, but did not affect the precipitating ability

Periodate oxidation of the polysaccharide A preparation led to a complete loss of the serological activities, both the precipitating and the sensitizing abilities disappeared

Neither tryptic nor carboxypeptidase digestions had any influence on the precipitating ability, whereas the sensitizing ability was completely lost

After one hour's incubation the carboxypeptidase digest was shaken with ion-exchange resin (Amberlite IR 120), and the washed resin was subsequently extracted with aq 5 N ammonia (5) Chromatography of the aq-NH_3 extract after removal of the ammonia, revealed the presence of all the amino acids found in the hydrolysate of polysaccharide A, *i e* lysine, glutamic acid, glycine, serine, and alanine After two additional digestions with carboxypeptidase no further release of amino acids could be observed The material that had been digested with carboxypeptidase was passed through a Dowex 50 column, and the fractions which gave the polysaccharide A line on agar gel diffusion were collected On hydrolysis and paper chromatography, this eluate could be shown to contain all the amino acids mentioned above

Examination for free amino acids after half an hour's incubation with carboxypeptidase showed only alanine and glycine, which by visual comparison seemed to be present in approximately equal amounts

Chromatographic examinations for DNP-amino acids, after treatment

with DNFB and subsequent hydrolysis (5), showed DNP-alanine and a trace of ε DNP lysine

The phenyl iso thiocyanate method (5) released PTH alanine as demonstrated by paper chromatography Repeated PITC treatment of the residual material did not reveal any further PTH-amino acids

No release of free amino acids could be observed on mild alkaline hydrolysis This shows that the polysaccharide A preparation does not contain ester linked amino acids, since ester-linked alanine in teichoic acid is completely released upon such hydrolysis (9)

DISCUSSION

Periodate oxidation and digestion with β N acetylglucosaminidase led to degradation of the teichoic acid structure of polysaccharide A with resultant destruction of its precipitating ability No catalysts were used in the diazomethane treatments It could therefore be assumed that only the phosphate groups were methylated by this reagent The fact that this alteration led to a loss of the precipitating ability, might suggest that the phosphate groups of teichoic acid are specifically involved in the antigen antibody linkage Acid phosphatase, however, releases phosphoric acid without affecting the precipitating ability, thus indicating that the terminal phosphate groups are not involved in the activity This problem calls for further investigations

Polysaccharide A has been shown to be a constituent of the staphylococcal cell wall (9) Consequently, the mucopeptide moiety of polysaccharide A would be expected to have a structure similar to that of the mucopeptide of cell wall preparations Several investigations of mucopeptides from bacterial cell walls have been reported (2, 14, 3, 4, 16) Generally mucopeptides are probably made up of N-acetylglucosaminyl N acetylmuraminyl fragments joined to peptide moieties by an amide bond between the carboxyl group of N acetylmuramic acid and an amino group of an amino acid of the peptide Also the existence of N O di acetylmuramic acid in cell wall preparation from *Staphylococcus aureus* has been reported (4, 16) It has been suggested (2) that a dimerization of the saccharide peptide entities occurs by a linkage between an ε NH_2 group of lysine and an α COOH group

It has previously been shown by absorption experiments (12) that the teichoic acid moiety of polysaccharide A which gives rise to a specific precipitation line on agar gel diffusion, is not involved in the ability to sensitize tanned sheep cells The effect of modifications of the functional groups of the peptide together with the enzymic digestion strongly suggest that the peptide moiety is responsible for the sensitizing ability of polysaccharide A However, the fact that the sensitizing activity was destroyed by periodate oxidation as well, indicates that the amino sugars also are involved in this activity

The carboxypeptidase experiments showed that the digestion of the

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Examination for free amino acids after half an hour's incubation with carboxypeptidase showed only alanine and glycine, which by visual comparison seemed to be present in approximately equal amounts

Chromatographic examinations for DNP-amino acids, after treatment

by β phenyl N acetylglucosamine He suggested that the β-linked N-acetylglucosamine of his teichoic acid might be responsible for the sensitizing ability It seems likely that his teichoic acid preparation was contaminated with some mucopeptide

SUMMARY

The chemical basis for the ability of polysaccharide A to sensitize tanned sheep erythrocytes to agglutination has been studied

Polysaccharide A was subjected to reaction with 2,4-dinitro-1-fluorobenzene (DNFB), phenyl-*iso*-thiocyanate (PITC), diazomethane, to periodate oxidation and to digestion with trypsin, carboxypeptidase-A, and β N acetylglucosaminidase

The experiments showed that the sensitizing ability of polysaccharide A is due to the mucopeptide moiety It seems likely that the muco peptide of polysaccharide A includes both saccharide-peptide complexes with free C terminal and peptides cross linked between N-acetyl amino sugar chains, and most probably only the former are capable of sensitizing tanned sheep cells

Both the DNFB and PITC methods showed N-terminal alanine, whereas digestion with carboxypeptidase-A for detection of C-terminal residues did not give conclusive results

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peptide was only partial, which suggests the existence of peptide chains with both free and bound C-terminal amino acids Most likely, only the open chains participate in the serological activity The peptides that are not digested with carboxypeptidase are probably cross-linked between N-acetyl amino sugar chains The application of carboxypeptidase digestion for identification of the C-terminal residues have not always been successful (14, 5) Since both alanine and glycine were released after half an hour's incubation with carboxypeptidase, it cannot be determined which of the two is C-terminal

Although the peptide is assumed to be linked to the carboxyl group of muramic acid (2), N-terminal alanine was detected Since no ester-linked alanine could be observed after mild alkaline hydrolysis, the released N-terminal alanine must be bound to the peptide by an amide linkage.

As suggested by *Salton* (14), N-terminal groups of mucopeptides are expected to arise only when branching and cross-linking occur with amino acids such as lysine or diamino pimelic acid Thus, in polysaccharide A, lysine may carry single amino acid substitutes (alanine) or peptides (with N-terminal alanine) attached to the ε-NH_2 group However, no further release of PTH-amino acids indicates single alanine substitution Since a trace of ε-DNP-lysine could be observed, it seems likely that the ε-NH_2 groups of lysine in our polysaccharide A preparation are both free and substituted or cross-linked

Since the mucopeptide of polysaccharide A seems to be responsible for the ability to sensitize tanned sheep cells, it would be expected that cell wall preparations, from which teichoic acid has also been removed, have this ability However, as indicated by the carboxypeptidase experiments, probably only saccharide—peptide complexes with open peptide chains are capable of sensitizing tanned sheep erythrocytes

Absorption experiments have shown (12) that tanned sheep cells sensitized with polysaccharide A remove all haemagglutinating antibodies from the sera, whereas tanned cells sensitized with protein A did not seem to remove the agglutinating antibodies directed against polysaccharide A sensitized tanned cells Protein A has been shown not to contain polysaccharides and the serological difference, as evidenced by absorption, may be related to the amino sugars of polysaccharide A The same amino acids were found in the sensitizing fragments of both preparations, and periodate oxidation of protein A does not alter the haemagglutination titre

The proportion of amino acids in mucopeptides may be similar for certain groups of bacteria (13, 15), but the sequence and arrangement of these amino acids may differ widely Thus small variations in the arrangement may poss
perties or specificities
nature of the glycosidic linkage of the saccharide chain *Morse* (11) re-
ported inhibition of the haemagglutination by N-acetylglucosamine and

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INFECTIONS WITH TICK-BORNE ENCEPHALITIS VIRUS IN THE SWEDISH POPULATION OF THE ELK (ALCES A. ALCES)

By

ARNE SVEDMYR, GEROLF VON ZEIPEL, KARL BORG
and HANS-JORGEN HANSEN

Received 8 vii 65

Many animal species have been reported to participate in the circulation of tick borne encephalitis (TBE) viruses in nature (for references see 1) The largest of the European deer, the elk (*Alces a alces*), does not seem to have been investigated previously from this point of view despite the fact that the elk is a very common forest animal in northern Europe Thus the Swedish elk population was estimated to be approximately 150,000 animals at the beginning of the hunting season of 1962 during which 31 298 elks were reported to be shot Actually the number of domestic cows in Sweden, which has a largely milk-drinking human population is only about 10 times the number of elks As the elk thus may be of considerable importance for the circulation of tick-borne virus we have carried out a limited survey of neutralizing antibodies among Swedish elks and have also made a successfull attempt at virus isolation

MATERIALS AND METHODS

Collection of Blood Samples

During the hunting season in the autumn and winter of 1962 hunters of a few geographical regions were requested to collect blood specimens for laboratory investigations Specimens from a total of 75 elks were obtained The distribution of these elks as to county of origin is given in Table 1 Sera were inactivated at 56° C for 30 min and stored at a temperature of about —25° until tested

Tissue Culture Methods

[illegible]

discarded in 1960 due to a mycoplasma infection Reserve lines kept in our laboratory and daughter lines requested back from Helsinki (Dr N *Oker Blom*) and Vienna (Dr *H Moritsch*) as well as two other lines kindly supplied by Dr *G S Stalberg* Detroit Mich all proved to be somewhat different from each other as to appearance of the cells and unfortunately to give only a poor and unreliable cyto

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RESULTS

1 *Serological Survey*

The sera of not less than 33 out of the 75 elks investigated neutralized HYPR virus presumably as a result of a previous TBE virus infection

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TABLE 1

Neutralizing Antibodies among Elks and Domestic Cows

County (official symbol)	Part of Sweden	Frequency of immune animals Elks (1962)	Cows (1958)
X	Just north of endemic zone	6/30	0/98
D	southeast	11/13	12/45
G	(= endemic zone)	1/1	15/44
H		10/14	62/130
L		2/4	10/65
P + R	West	3/8	9/295
AC	North	0/5	1/136

Nominator number of animals with antibodies
Denominator number of animals investigated

2 *Isolation of TBE Virus from the Blood and Brain of an Elk Calf Suffering from Encephalitis*

On June 15 1962 a lumberjack at Tunaberg Sodermanland (about 60 miles southwest of Stockholm) observed an elk cow with two calves ... calves a male one kept walking in ... ported to the forester who a couple ... alone and still walking around at the same place occasionally falling but then continuing his circular way The eyes appeared covered with an opaque membrane and the animal did not close its eyes to the approaching hand nor to flies Although apparently blind the calf seemed to react to sound and smell It was shot through the heart

At autopsy performed three days later there were moderate post mortem changes The calf weighed 18 7 kg The only gross lesion observed in addition to the changes caused by the shot was a slightly greyish opacity of the cornea of the right eye

Specimens from various organs were taken for histological examination Thus material from various parts of the cerebral cortex the brain stem the cerebellum and the medulla oblongata in the neighbourhood of the pons was examined as were the eyes and parts of the liver and kidneys

In all sections of the brain specimens although more pronounced in

pathic effect after infection with louping ill or HYPR viruses A third subline of our cells which had been maintained by Dr *L Lycle* in Gothenburg showed a complete degeneration after infection with these viruses however and has therefore proven even more suitable for neutralization tests than the original line It has kept these characters during the past 4 years Of the lines of Detroit 6 cells mentioned only one of those obtained from Dr *Stulberg* has ever been cloned

Neutralization Tests on Elk Sera

Sera were tested undiluted (with the exception of two positive specimens which were diluted 1 2 and a third one 1 5) against 10 100 TCD_{50} of HYPR virus as judged from simultaneous virus titrations All positive specimens however were tested or retested against 30–100 TCD_{50} Virus serum mixtures were incubated at 37° C for 1 hour followed by +4° C overnight before being inoculated each into two stationary tube cultures of Detroit 6 cells

Other details about the technique used may be be found in a previous paper (4)

The HYPR strain of the western type of tick borne encephalitis virus had been kindly supplied by Dr *D Blaškovič*, Bratislava, and had undergone 44 mouse brain passages followed by 5 or 6 passages in Detroit 6 cells (3)

Techniques Used for Isolation and Identification of Virus

Virus was isolated from materials obtained at the autopsy of an elk calf Half the brain was sent to the virus laboratory, the other half used for histological investigations The specimen was prepared for inoculation in the following way In order to disinfect the surface of the specimen slices of various parts of the brain about 3 4 cm thick were placed on a wire mesh of stainless steel which was then dipped for a moment in ethanol and thereafter set aflame thus burning the surface The outer layer was removed and [illegible] prepared by grinding with sand and [illegible] of penicillin and 200 μg of streptomyc[illegible] d centrifugation 0 02 ml of the extrac[illegible] three weeks old mice

Haemolysed serum separated from a blood clot [illegible] calf was tested in the same way as the brain e[illegible] the mice within two days the serum was retested [illegible] buffered saline

The brains of the mice were harvested when the animals were sick or moribund A 10 per cent extract was prepared by disintegration with phosphate buffered saline in a Servall omnimixer The extract was clarified by low speed centrifugation and then stored at —70° C

Complement fixation and neutralization tests were used for typing of isolated viruses as described previously (5) Ten per cent mouse brain extracts from the isolation experiments provided the virus materials As positive control antigens in the CF test we used a 10 per cent brain extract from mice succumbing to HYPR virus infection as well as our routine HYPR tissue culture antigen inactivated by β propiolactone (3)

For CF tests we employed a pooled convalescent serum from patients with TBE as well as the serum from guinea pigs given 2 intraperitoneal injections of 1 0 ml of undiluted HYPR virus from Detroit 6 cultures 1 5 months apart and bled 8 days later For NT we used serum from guinea pigs immunized with the 10 per cent brain extract from mice inoculated with HYPR virus here a scheme of 0 3 ml intravenously followed by 1 0 ml intramuscularly after 4 months and bleeding 9 days later was used Sera were heated at 56° C for 30 minutes before use

CF tests were performed with a micro technique (2) In the neutralization test tenfold serial dilutions of virus were mixed with the same volume of undiluted serum Following incubation at +4° C over night 0 1 ml amounts of each mixture were inoculated intraperitoneally into each of 5 three weeks old mice

Histological T[illegible]hn[illegible]que

Specimens [illegible] 10 per cent water solution of [illegible] n or according to van Gieson [illegible] or with methyl green pyronine (*Unna Pappenheim*)

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Figs 1 4

Fig 1 0 3781/62 Lll calf Destruction of Purkinje cells with neuronoj hagia an l reactive ghosis in the cerebellar cortex H L 150 ×
Fig 2 0 3781/62 Llk calf Cerebellum Demyelinization of the white matter Mahor 150 ×
Fig 3 0 3781/62 Elk calf The brain stem I erivascular lymphocytic cuffing and ghosis with neur noj hagia H I 300 ×
Fig 4 0 3781/62 Flk calf Cerel rum Lymj hocytic and j lasmacellular infiltrates in the meninges H L 500 ×

the white matter of the cerebellum the brain stem and the medulla oblongata there were diffuse or more circumscribed areas of demyelin ization The nerve cells especially the Purkinje cells showed different degrees of chromatolysis and destruction with neuronophagia Reactive ghosis also appeared in connection with demyelinization Vascular reac tions consisted of swelling of the endothelial cells perivascular oedema



Fig 5
0 3/81/62 Elk calf The right eye showing intracellular oedema with exfoliation in the cornea The left eye (b) reveals a normal cornea H E 300 ×

and perivascular cuffings with large immature lymphocytes and strikingly often plasma cells Such infiltrates were seen occasionally in the meninges as well

The external half of the epithelium of the right cornea appeared swollen due to an intracellular oedema In the same outer layer there was also some exfoliation and vesicles, yet without signs of inflammation

P A D *Nonpurulent meningo encephalitis*

Specimens from brain, liver and spleen were cultured for bacteria particularly Listeria No specific bacterial infection was disclosed

However, a strain of TBE virus was isolated from the blood specimen as well as from the brain extract Three weeks old mice inoculated with serum diluted ½ fell ill 8 days after inoculation, those inoculated with the 20 per cent brain extract already after 6 days The original elk brain extract contained only 2 LD_{50} per 0 02 ml when retested after two years of storage at —70° C (mice becoming ill after 9–10 days) whereas no virus was by then recovered from a 1 4 dilution of the serum specimen In an intraperitoneal test in mice (Table 2) 0 05 ml of undiluted serum

TABLE 2

Intraperitoneal Mouse Neutralization Test on Virus Recovered from Elk Brain

Virus titre (LD_{50} per 0 05 ml) in presence of	
Hyperimmune serum*	Normal guinea pig serum
10°	$10^{5.1}$

* Serum from guinea pigs hyperimmunized with mouse brain HYPR virus

TABLE 3

Identification of Virus Strains by CI Tests

Antigen	Dilution of antigen	Dilution of antiserum								
		Hyperimmune serum*					Human convalescent serum			
		32	64	128	256	512	32	64	128	256
Mouse passage 1 of elk brain	4	+	+	+	±	tr	+	+	+	±
	8	+	+	+	±	tr	+	+	±	tr
	16	+	+	+	tr	tr	±	±	tr	—
	32	+	±	tr	tr	tr	tr	—	—	—
	64	—	—	—	—	—	—	—	—	—
Mouse passage 1 of elk serum	4	+	+	+	±	—	+	+	+	+
	8	+	+	+	+	tr	+	+	+	+
	16	+	+	+	+	tr	+	+	+	±
	32	+	+	+	±	tr	±	±	tr	—
	64	+	±	tr	—	—	—	—	—	—
Elk brain	1	—	—	—	—	—	—	—	—	—
HYPR mouse brain antigen	4	+	+	±	—	—	+	+	±	tr
	8	+	+	+	—	—	+	±	tr	—
	16	+	+	±	—	—	±	tr	—	—
	32	±	±	tr	—	—	—	—	—	—
HYPR tissue culture antigen	4						+	+	+	±
	8						±	tr	tr	tr
Normal mouse brain	1	—	—	—	—	—	—	—	—	—

* Serum from guinea pigs hyperimmunized with HYPR tissue culture virus

tr indicates the presence of traces of unhaemolyzed cells

from guinea pigs hyperimmunized against HYPR virus neutralized $\geq 10^{5.1}$ LD_{50} of the agent obtained from calf brain A pool of human convalescent sera after TBE reached similar CF titres against the two isolates as against HYPR antigens whether these were prepared in mice or in tissue culture (Table 3) The brain extracts gave slightly higher antigen titres against serum from guinea pigs hyperimmunized against HYPR virus than against convalescent serum

The original extract of the elk brain apparently contained too little CF antigen to give a positive reaction

DISCUSSION

Although comparisons between the present survey of TBE antibodies in elks and our 1958 investigation of domestic cows (4) should be made with some caution, due to the small and unevenly scattered elk material and particularly to the fact that the neutralization tests this time were somewhat more sensitive (smaller virus dose employed), the differences appear sufficiently marked (Table 1) to suggest that elks may be exposed to infected ticks more often than are domestic cows This conclusion is strengthened by the finding of a significant number of positive elks just outside the region judged as "endemic" in the cow study in the wide forests of the X county in the north and in the national forest park at Hunneberg on the border of counties P and R in the west

hbourhood of

immune elks

the infection

between 1958 and 1962, appear less likely

It may be recalled that in our previous study (4) cows grazing solely on cultivated pasture had NT antibodies less frequently than animals from the same districts grazing at least partly on natural pastures containing bushes and trees The fact that elks in southern Sweden certainly spend most of their time in areas the ecology of which favors the development of Ixodes ricinus ticks may probably account for a higher rate of NT antibodies to TBE virus

TBE virus of the western type anyway, is apparently nonpathogenic for most mammals (1) Very probably it is little pathogenic also to elks as so many of these animals obviously survive the infection The present finding of TBE virus in the brain and blood of an encephalitic elk calf is of interest from this point of view Although it definitely proves that elks can be infected with this virus it cannot be concluded with absolute certainty that the TBE virus actually caused the encephalitis Our data do not supply any significant evidence for a local proliferation of virus in the brain On the other hand, the histopathological picture revealed features characteristic of a viral inflammation in general Moreover, some features, e g the nerve cell involvement, could well fit in with the picture of a tick borne encephalitis The eye lesion observed was non-

inflammatory with no relation to *e g* the panophtalmitis seen in bovine malignant catarrh

Most studies on the pathogenicity of TBE virus for various animals have been performed on adult animals The present observations suggest that studies on very young mammals may sometimes be rewarding

It seems probable that the elk plays a role in the circulation of TBE virus in nature It is not known however, if the virus titres reached in the blood are high enough for infection of biting ticks

SUMMARY

Elks (*Alces a alces*) from the endemic region of southeastern Sweden have neutralizing antibodies to TBE virus strikingly often probably more often than have domestic cows A strain of TBE virus was isolated from the brain and blood of an encephalitic elk calf

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TRANSDUCTION OF THE MU-FACTOR IN *ESCHERICHIA COLI*

By

WENCHE BLIX GUNDERSEN

Received 21 vii 65

Streptomycin resistance resulting from the action of an episomelike mutator factor in *Escherichia coli* has for some time been the subject of investigation in this laboratory (5, 3) The mutator streptomycin resistance is a high level resistance It segregates like a chromosomal marker in bacterial cross, but its location seems to be distinct from that of ordinary streptomycin resistance, possibly clockwise between the streptomycin locus and the threonine region Ordinary streptomycin resistance is a recessive mutation (7) Genetic analyses have revealed that the determinant for streptomycin sensitivity is present in the mutator resistant cells, thus this resistance seems to behave like a phenotypically dominant character It has been suggested that the mutator resistance is the result of an attachment of the mutator factor to the bacterial chromosome, possibly at the site of a suppressor or a regulator locus resulting in an interference with the expression of the streptomycin gene (3) It was of some interest to see whether the mutator resistance is dominant in the sense that it is expressed immediately upon entering the cell, like a dominant, structural gene, or whether it would need time for phenotypic expression, like for instance the i gene of the β galactosidase system, which is also a dominant gene (9) The present paper reports transductions performed with strains harbouring the mutator factor In these studies the resistance resulting from the action of the factor has been compared with the ordinary streptomycin resistance

MATERIALS AND METHODS

Media Heart Infusion Broth and Agar (Difco) were used as liquid and solid complete media The minimal medium was that of *Davis & Mingioli* (2) Vitamins and amino acids were kept in sterile solutions and added to the media when necessary

[illegible]

The phage P1kc was a gift from dr *H P Treffers* The strain K12 T71Mu was prepared in this laboratory by infection using *E coli* 635Mu as donor (5)

Preparation of streptomycin resistant mutants Streptomycin resistant mutants were selected on complete media containing 100 μg streptomycin per ml

Preparation of phage stocks The phage stocks were prepared as described by *Lennox* (8) The phage Plkc was propagated on the strains T71 str r, K12 1M str r (called Treffers str r), T71Mu str r and T71Mu, and a control phage stock was prepared on the strain T71 thr

Transductions The transduction experiments were carried out according to the procedure of *Lennox* (8) and of *Yanofsky & Lennox* (13) The recipient in the majority of the experiments was K12 T71 thr Recombinants were selected for transduction to prototrophy and to streptomycin resistance, the transduction from auxotrophy to prototrophy serving as the control in each case (13) The following controls were carried out in each experiment

1 Sterility tests of all the ingredients
2 Transduction with phage lysate propagated on the recipient strain
3 Test for mutation to prototrophy and streptomycin resistance of the recipient strain

The selective plates were minimal plates for selecting recombinants to prototrophy and minimal plates plus growth factor to which 100 μg of streptomycin per ml was added either immediately or after incubation for phenotypic expression had taken place (1) Minimal threonine plates containing 15 μg of streptomycin per ml was used for selection of low level streptomycin resistant transductants

RESULTS

Transduction of High-Level Streptomycin Resistance

Preliminary experiments indicated that no recombinants are present when the transduced cells are plated directly on plates containing 100 μg of streptomycin per ml Thus, if a phage-mediated transfer of the chromosomal marker for mutator streptomycin resistance takes place, this marker will not protect the cells from the cidal action of 100 μg of streptomycin per ml immediately after introduction into them

TABLE 1

Results from Transduction Experiments Selecting for Recombinants Resistant to 100 μg of Streptomycin per ml with and without Time Allowed for Phenotypic Expression

Donor	thr⁻→thr	Selection for str r directly—no time for phenotypic expression	Selection for str r allowing 6 hours for phenotypic expression	str r/thr⁺ per cent
T71Mu str r	4418	0 <0 02 %	701	16
T71Mu	7925	0 <0 01 %	1044	13
T71 str r	5107		2005	39
Treffers str r	4733		1706	36

The results are the sum of several independent experiments
The recipient in all the experiments is T71 thr

Some results recorded in Table 1 show, as expected, that ordinary streptomycin resistance is transduced into sensitive recipients when time is allowed for phenotypic expression The ratio of streptomycin

resistant recombinants to prototrophic ones is fairly constant from one experiment to the next, probably just reflecting the difference of integration of the different markers When the same time was allowed for phenotypic expression using T71Mu str-r as the donor, recombinants appeared on the *threonine-streptomycin* plates The ratio of the streptomycin resistant recombinants to the prototrophic ones, however, seemed consistently lower, suggesting that the recombinants were due to the transfer of the mutator factor with a possible subsequent modification of the recipient chromosome Experiments were accordingly carried out using T71Mu as the donor, resulting in the appearance of recombinants on the threonine-streptomycin plates, with the same low ratio str r thr+ This is taken to indicate that it is the mutator factor *and not the mutator strepomycin resistance* which is transferred to the sensitive recipients when T71Mu str-r and T71Mu are used as donors

Examination of the Streptomycin Character of the Recombinants

Cells that have become streptomycin resistant as a consequence of the action of the mutator factor also carry the mutator factor in the infectious form (5) Thus phenotypically similar streptomycin resistant clones *may be distinguished by looking for their ability to infect a* mutator negative cell Theoretically exceptions to this rule may occur With this purpose recombinant clones growing on threonine-streptomycin plates were examined When the donor was of the ordinary streptomycin resistant type, the recombinants were also non-infective, streptomycin resistant organisms When the phage stock had been propagated on T71Mu str-r or T71Mu the recombinants were all found to carry the mutator factor in the infectious form Upon further investigation it was found that the recombinants which had been selected on threonine streptomycin plates containing 100 μg of streptomycin per ml were not resistant to 100 μg of streptomycin per ml when transferred *to a complete medium* The cells carried the mutator factor in the extrachromosomal state, their chromosome was not modified to give high level streptomycin resistance Thus the fact was revealed that the presence of the mutator factor in its extrachromosomal state protects the cells against the lethal effect of streptomycin under certain conditions The results from investigation of this problem are reported elsewhere (4) The present experiments show that it is possible to transfer the mutator factor from a mutator or a mutator-streptomycin-resistant cell to a mutator-negative cell by means of transducing phage as well as by infection The frequency with which the mutator factor may modify the bacterial chromosome seems to be the same, of the order of 10^{-4} regardless of its *origin and mode of transfer* The transductants which have received the mutator factor are able to transfer it to negative cells by infection

Transduction of Low-Level Streptomycin Resistance

Growth experiments in presence of streptomycin indicate that the presence of the mutator factor in the cell will protect the cell against low concentrations of streptomycin regardless of the physiological state of the cells at the time of exposure (4) It was of some interest to elucidate the time when this low-level streptomycin resistance becomes genetically expressed Accordingly transduction experiments were carried out with the application of a slightly different selection technique After treatment with the transducing phage, the cells were plated directly on threonine plates containing 15 µg of streptomycin per ml, as well as on minimal plates The results of such experiments are recorded in Table 2 When ordinary streptomycin resistant strains were used as donors virtually no recombinants were obtained on the streptomycin plates, as expected (14, 12) When the mutator strains were donors, however, a fairly high number of recombinants appeared on the streptomycin-containing selective plates No time was allowed for phenotypic expression in this system Thus it seems as if the mutator factor gives an immediate protection against the lethal action of streptomycin from the moment when it enters the sensitive recipient

TABLE 2
Transduction to Low Level Streptomycin Resistance Allowing no Time for Phenotypic Expression

Donor	thr →thr⁺	Streptomycin 15 µg per ml	Ratio in per cent
T71 str r	2170	12	0 5
Treffers str r	2885	5	0 17
T71Mu	4685	961	20
T71Mu str r	1141	273	24
	657*	131*	20*

* arg →arg⁺

DISCUSSION

The present investigation has revealed the existence of a type of streptomycin resistance which seems to differ from the ones described earlier The episome-like mutator factor which has been the subject of earlier reports from this laboratory (5, 3) endowed on the cells a high "mutant" frequency to high-level streptomycin resistance This streptomycin resistance is expressed in cells possessing the normal gene for streptomycin sensitivity, and since the sensitivity gene actually is a dominant one (7) the mutator resistance may act by interference with the expression of this gene The mutator resistance segregates like a chromosomal marker (3), and it was suggested to be due to an attachment of the mutator factor to the bacterial chromosome, possibly acting like a suppressor gene Attempts to transduce the dominating,

high level streptomycin resistance and obtain immediate phenomic expression have failed Only the mutator factor is transduced by means of the phage into the recipient cells, and the mutator factor thus transferred seems to have the same chance of modifying the recipient chromosome (possibly by attachment) regardless of whether the transducing phage has been propagated on a mutator streptomycin resistant cell or a mutator cell, or whether the recipient cell has acquired the factor by infection It thus does not seem to 'remember" its specific site on the bacterial chromosome any better when it originates from a mutator streptomycin resistant cell, at least not to an extent which can be registered with our technique The frequency with which the mutator factor modifies the bacterial chromosome, resulting in high-level streptomycin resistance, is of the same order as the attachment of the F factor to the bacterial chromosome, giving rise to Hfr-strains, namely around 10^{-4} (6) But whereas the F factor can attach to several sites around the chromosome, the mutator factor only seems to have one site (3) Recombinants which have received the mutator factor by means of transducing phage are able to transfer it to negative cells by infection

During the present investigation it was found that the mutator factor itself, in its extrachromosomal state endows the cells with a low-level streptomycin resistance which is expressed immediately after the mutator factor has entered the recipient cell This streptomycin resistance differs from the types earlier described, ordinary one-step streptomycin resistance (7, 14, 12) as well as from the streptomycin resistance carried by the RTF-factor (11) The RTF-streptomycin resistance when transduced into a recipient cell needs a phenomic lag of 90 minutes before addition of streptomycin It is, however, expressed before the first cell division, suggesting it is not recessive (10) The particular streptomycin resistance of the cells carrying the mutator factor in its extrachromosomal state will protect the cells against quite high concentrations of streptomycin, at least 200 µg per ml when they are growing in minimal medium (4) There is reason to believe that the apparent need for phenomic lag when selecting for recombinants with 100 µg of streptomycin per ml, as was done in the experiments listed in Table 1, is not an actual phenomic lag allowing time for integration and segregation of a genetic marker but rather a change in the physiological condition of the cells

SUMMARY

Transduction technique has been applied as an attempt to further elucidate the genetic nature of the streptomycin resistance resulting from the action of the mutator factor in *E coli* High level mutator streptomycin resistance is *not transduced* into sensitive recipients, in contrast to ordinary, one step streptomycin resistance The mutator

factor, however, is transferred by the tranducing phage into sensitive recipients The resulting transductants have the characteristics indicating that they possess the mutator factor in its extrachromosomal state, regardless of the donor being a mutator or a mutator streptomycin resistant cell

The mutator factor, when introduced into a sensitive recipient confers on it resistance to low concentrations of streptomycin This resistance is expressed immediately, with no need for phenomic lag The resistance appears to be of a dominant nature, and the level of restistance depends upon the physiological state of the cells

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REDUCED STREPTOMYCIN KILLING IN E. *COLI* CARRYING THE MU-FACTOR IN ITS EXTRACHROMOSOMAL STATE

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Studies which were based on transductions with subsequent analyses of the recombinants revealed that cells of *E coli* carrying the mutator factor (14) in the extrachromosomal state are resistant to low concentrations of streptomycin (12) This resistance is of a dominant nature, since it is expressed immediately after the mutator factor has entered a formerly sensitive cell *It also seems that the level of resistance* varies with the composition of the medium in which the cells are growing This particular, dominant, low-level insensitivity seems to differ from the types of streptomycin resistance previously known The one-step, high level streptomycin resistance seems to be well established genetically (20 21, 19, 18, 32) as well as physiologically (28, 4, 5, 6) Other types of low-level streptomycin resistant organisms have been described, in *Salmonella typhimurium* (33) and in Pneumococcus (3, 26) In some instances (33) the resistance was combined with slow growth The *genetic determinant of such low level resistance* has been located on the bacterial chromosome, distinct from the location of the ordinary streptomycin locus (3, 26, 33)

In most of the work concerned with streptomycin resistance, the mutants have been selected as well as characterized on complete media containing streptomycin However, *Tzagoloff & Umbreit* (29) who examined the influence of streptomycin on the nucleotide excretion by a streptomycin sensitive strain of *E coli* B, found that the amount of streptomycin required to inhibit growth varied markedly with the medium When growing in nutrient broth, *E coli* B was completely inhibited by 0 1 μg of streptomycin per ml whereas when rapidly growing in minimal medium as much as 20 μg was needed to inhibit growth i e 200 times as much The authors suggest that this difference is due to the salt and phosphate content of the medium

The streptomycin resistance carried by the RTF-factor seems to have many characteristics in common with the low level streptomycin resistance of the mutator factor When listing the average resistance of

the various strains to different drugs, *Watanabe & Fukasawa* (31) *report that Salmonella typhimurium* LT2 carrying the RTF-factor is resistant to between 5 and 10 μg of streptomycin per ml in complete medium and between 100 and 500 μg per ml in minimal medium The phenomenon is not further commented upon

The present paper deals with the influence of growth conditions on the phenotypical expression of the previously described (12) low level streptomycin resistance shown by cells possessing the mutator factor of *E coli* in its extrachromosomal state

MATERIALS AND METHODS

Bacterial strains The strains K12 T71 and T71Mu have formerly been described (14, 11, 12)

Media Heart Infusion Broth and Agar (Difco) was used as complete medium The *minimal medium* was that of *Davis & Mingioli* (7), the low K medium that of *Dubin & Davis* (8) Yeast extract and Casein hydrolysate (Difco) were kept in sterile solutions of 8 per cent Glucose, in a final concentration of 0 5 per cent, was usually the source of carbon and energy When histidine or glutamate was used instead the final concentration was also 0 5 per cent

Viable count For determination of the viable count the cultures were spread in dilutions on complete plates

OD measurements Cultures growing in side-armed flasks at 37° C with shaking were measured in a Beckman Model C Colorimeter with the green filter, having an abs max of 524 mμ

RESULTS

The reaction of the strain K12 T71Mu to streptomycin when growing in media of different composition has been shown in Fig 1 When growing in complete medium, the addition of 100 μg, of streptomycin per ml is followed by a rapid decrease in the viable count, 90 per cent of the cells are dead within the first hour When the cells are growing in minimal medium, however, the growth is apparently quite unaffected by the addition of streptomycin This phenomenon is further illustrated in Fig 2 and Fig 3 When the OD is followed as in Fig 3, a slight retardation of the growth can be seen when the culture has reached the exponential phase The method based on viable count does not seem sufficiently sensitive to record this small difference between the growth rate of T71 Mu in minimal medium and in minimal with streptomycin

That this particular streptomycin resistance is indeed due to the presence of the mutator factor is seen in Fig 4 T71 Mu was prepared in this laboratory by infection of the strain K12 T71, a wild type K12 (14) The lethal effect of streptomycin on the original strain T71 is pronounced, within one hour there is a decrease in the viable count of 99 9 per cent

In Fig 5 an attempt has been made to elucidate the significance of the physiological state of the cells at the moment of exposure to streptomycin Culture I and IV confirm the results shown in Fig 1 The cells in Culture II were actively growing in complete medium before the

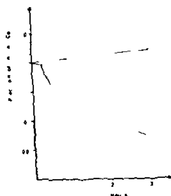


Fig 1

rowing in medium
omycin to a final
growing cultures

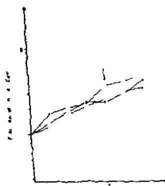


Fig 2

Effect of streptomycin on the viable count in cultures of T71Mu growing in medium A glucose

●——● Control without streptomycin
✗——✗ Streptomycin added at zero time
○——○ Streptomycin added after 2 hours

exposure to streptomycin The metabolism apparently comes to a halt *followed by a lag period in which streptomycin has no effect Other* experiments indicate that after a long lag 4–5 hours growth resumes unaffected by the presence of streptomycin Culture III which had been pregrown in minimal medium and then exposed to streptomycin in complete also seems to have a lag period before the killing of streptomycin becomes as rapid as is usual in complete medium The reason for this delay is not known

Tzagoloff & Umbreit (29) report that the difference in the amount of streptomycin required to inhibit growth of streptomycin sensitive

the various strains to different drugs, *Watanabe & Fukasawa* (31) *report that Salmonella typhimurium* LT2 carrying the RTF-factor is *resistant to* between 5 and 10 µg of streptomycin per ml in complete *medium and* between 100 and 500 µg per ml in minimal medium The phenomenon is not further commented upon

The present paper deals with the influence of growth conditions on *the* phenotypical expression of the previously described (12) low-level *streptomycin* resistance shown by cells possessing the mutator factor of *E coli* in its extrachromosomal state

MATERIALS AND METHODS

Bacterial strains The strains K12 T71 and T71Mu have formerly been described (14 11, 12)

Media Heart Infusion Broth and Agar (Difco) was used as complete medium The *minimal medium* was that of *Davis & Mingioli* (7), the low-K medium that of *Dubin & Davis* (8) Yeast extract and Casein hydrolysate (Difco) were kept in sterile solutions of 8 per cent Glucose, in a final concentration of 0 5 per cent, was usually the source of carbon and energy When histidine or glutamate was used instead the *final concentration* was also 0 5 per cent

Viable count For determination of the viable count the cultures were spread in *dilutions* on complete plates

OD measurements Cultures growing in side armed flasks at 37° C with shaking were measured in a Beckman Model C Colorimeter with the green filter, having an abs max of 524 mµ

RESULTS

The reaction of the strain K12 T71Mu to streptomycin when growing in media of different composition has been shown in Fig 1 When growing in complete medium, the addition of 100 µg, of streptomycin per ml is followed by a rapid decrease in the viable count, 90 per cent of the cells are dead within the first hour When the cells are growing in minimal medium, however, the growth is apparently quite unaffected by the addition of streptomycin This phenomenon is further illustrated in Fig 2 and Fig 3 When the OD is followed as in Fig 3, a slight retardation of the growth can be seen when the culture has reached the exponential phase The method based on viable count does not seem sufficiently sensitive to record this small difference between the growth rate of T71 Mu in minimal medium and in minimal with streptomycin

That this particular streptomycin resistance is indeed due to the presence of the mutator factor is seen in Fig 4 T71 Mu was prepared in this laboratory by infection of the strain K12 T71, a wild type K12 (14) The lethal effect of streptomycin on the original strain T71 is pronounced, within one hour there is a decrease in the viable count of 99 9 per cent

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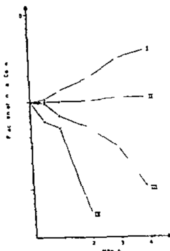


Fig 5

Effect of streptomycin on T71Mu in various physiological states
Cultures I and III were actively growing in medium A glucose at 37° C with shaking, they were diluted and inoculated into prewarmed
I medium A glucose with streptomycin
III complete medium with streptomycin
Cultures II and IV were pregrown in complete medium and similarly diluted into prewarmed
II medium A glucose with streptomycin
IV complete medium with streptomycin

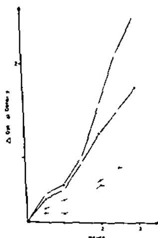


Fig 6

aining 200 μg
At zero time
flasks to one
s are recorded
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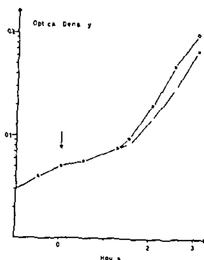


Fig 3

Effect of streptomycin on the growth of T71Mu in medium A-glucose as seen by OD readings Streptomycin to a final concentration of 100 µg per ml was added to one of the cultures (×——×), whereas the other culture (○——○) served as control



Fig 4

Protective effect of the mutator factor in the extrachromosomal state on cells growing in medium A glucose in the presence of 100 µg of streptomycin per ml
×——× T71 K12 (The wild type) ●——● T71Mu

E coli B in complete versus minimal medium was due to the salt and phosphate content of the medium Experiments carried out in order to elucidate this point are recorded in Fig 6 and Fig 7 T71Mu was growing in minimal medium to which was added increasing concentrations of casein hydrolysate or yeast extract The growth factors were added in small amounts from concentrated stock solutions in order to keep the salt and phosphate content as equal as possible The results seem to indicate that streptomycin is increasingly lethal with an enhanced growth rate

Plotz, Dubin & Davis (22) report that one or more of the salts in the

The minimal inhibiting concentration of streptomycin on the wild type, K12 T71 and on T71Mu when growing in complete and in minimal medium was determined in liquid culture by measuring the optical density, and on solid media by spreading the bacteria on plates containing increasing concentrations of streptomycin The results are listed in Table I

DISCUSSION

Several sites of action have been proposed for streptomycin, the cell membrane (1, 2, 17), *the nucleic acids* (23, 25) and the ribosomes (28, 27, 9, 4, 5, 6, 10) It seems that the evidence supporting the ribosomes as the site of action is accumulating, and this also seems to *encompass* comfortably the genetic findings of the system, the streptomycin sensitivity, resistance and dependence being three alleles of the same *cistron Some findings do not seem to fit this picture entirely, however,* suggesting the existence of at least one other mechanism of streptomycin resistance

Watanabe (30) suggests that the resistance to tetracycline, chloramphenicol and sulphonamides carried by the RTF-factor is due to a decreased permeability to each of the drugs About the streptomycin resistance carried by this factor very little is known, except that it is of a dominant nature *Rosenkranz* (24) *showed* that this streptomycin resistance must be located at a site other than the ribosome, and he postulates the mechanism to be one of altered permeability *Jacob, Brenner & Cuzin* (15) postulate that the F-factor, and possibly also other episomes like the RTF-factor, are located in connection with the *bacterial surface* in *their autonomous* as well as *their attached* state If their location is connected with the surface, which also is supported by other evidence (16), they may also be expected to interfere with the activities of this surface, turning it into a barrier for certain substances

The present findings concerning the behaviour of the streptomycin resistance carried by the mutator factor in its extrachromosomal state can be reconciled with the idea of a decreased permeability to streptomycin The results *seem to indicate that the presence of the mutator* factor protects the cells to a certain extent against the lethal action of streptomycin also when they are growing in complete medium, since the killing is not as rapid as in cells which *do not possess the factor* The results are also taken to indicate that the lethality of streptomycin in the *strain containing the factor* in its extrachromosomal *location* increases with the growth rate rather unspecifically The lethal effect does not seem to be influenced by the quantities present of particular salts Findings using another technique support these assumptions (13)

The effect of the Mu-factor in its extrachromosomal state is obviously *not* a low-level streptomycin resistance in the conventional sense, but rather a relative protection against the lethal effect of streptomycin

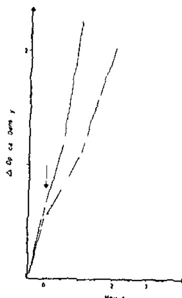


Fig 7

Effect of streptomycin on T71Mu growing in medium A glucose containing 200 µg (open circles) or 600 µg (closed circles) of yeast extract per ml At zero time each of the cultures were divided and distributed into two sided armed flasks to one of which was added streptomycin to give 100 µg per ml The controls are recorded with unbroken lines and the streptomycin containing cultures with dotted lines

minimal medium A interfere with the streptomycin uptake of the cells and *Rosenkranz & Carr* (25) report that high concentrations of phosphate interfere with the lethal effect of small concentrations of streptomycin on sensitive cells In contrast to these observations the effect of streptomycin on T71Mu when growing in a minimal medium of low phosphate content (8) could not be distiguished from that found in the ordinary minimal medium

The growth of T71Mu in medium A where the glucose had been replaced by either glutamate or histidine as the source of carbon and energy was not affected by the addition of 100 µg of streptomycin per ml

The effect of streptomycin was also examined under anaerobic conditions In complete medium, medium A-glucose and low-phosphate-glucose medium the addition of 100 µg of streptomycin per ml had the same effect on T71Mu as that found under aerobic conditions

TABLE 1

Streptomycin Concentrations Required to Give Complete Inhibition of Growth Listed in Microgram per ml

Strain	Complete	Medium A glucose
K12 T71	1 2	3 4
K12 T71Mu	10–15	200 225

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SUMMARY

The presence of the mutator factor of *E coli* in its extrachromosomal state protects the cells against the lethal action of streptomycin Factors increasing the growth rate of the cells diminish the protective effect of the mutator factor The protection may be due to a permeability barrier

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supplemented with 2 per cent of an 8 per cent solution of yeast extract (Difco), i e a final concentration of 1600 µg of yeast extract per ml Glucose, 0 5 per cent was added as carbon and energy source

Preparation of non proliferating suspensions Non proliferating suspensions were essentially prepared as described by *Engelberg & Artman* (6) Cells were harvested from exponentially growing cultures washed three times with cold saline on a membrane filter (Gottingen pore size 0 5 µ) and finally resuspended to a density 0 4 mg dry weight of cells per ml in a phosphate magnesium iron buffer (6) The optical density of the suspension was measured at 490 mµ in a Beckman spectrophotometer where an optical density of 0 4 roughly corresponds to 0 4 mg of bac ml (9)

pore fil [illegible] —0 15 µ pore size) and washed 4 times with 5 ml of [illegible]

identical samples of medium containing the same amount of 14C streptomycin and the value subtracted from those of the experimental samples The standard error of counting did not exceed 5 per cent The same procedure was used for the non proliferating suspensions

Chemicals Ten mg of 14C streptomycin ($CaCl_2$ salt) of a specific activity of 0 079 µc/mg of streptomycin base was a gift from dr *C Rosenblum* of Merck Sharp & Dohme Rahway N J U S A Owing to the low specific activity of the product it was used undiluted (One microgram of the 14C-streptomycin used in our experiments gave 52 counts per minute)

RESULTS

When the cells are grown in minimal medium under the conditions presented in Table 1 T71 is quickly killed, whereas T71Mu and T71Mu str r are quite unaffected (12) The absorbtion of streptomycin to the respective strains are not inconsistent with this phenomenon Besides adsorbing a greater amount of streptomycin, the sensitive strain seems to retain a larger part of it after washings with salt solution This may indicate that streptomycin is taken up by the sensitive strain, and not —or at least to a much smaller extent—by the two resistant ones

TABLE 1

Uptake of 14C Streptomycin by Cells Growing in Minimal Medium

Strain	Uptake of 14C-strept µg/mg dry weight bact	Strept left after 4 wash with salt sol
T71	3 58*	1 74*
T71 Mu	2 60*	0 28*
T71 Mu str r	2 84§	0 5†

For experimental conditions see materials and methods

* Mean of 5 independent experiments
§ Mean of 3 independent experiments
† Mean of 2 independent experiments

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UPTAKE OF ^{14}C-STREPTOMYCIN BY *ESCHERICHIA COLI* CARRYING THE MU-FACTOR

By

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Received 21 vii 6

Lately evidence has accumulated indicating that the lethal effect c streptomycin on sensitive cells is due to its interference with the readin of m-RNA on the ribosome, resulting in the formation of aberran protein (16, 7, 8, 3, 2, 10) It seems like resistance to or dependenc upon streptomycin is due to alterations in the structure of the ribo somes Microorganisms carrying the "Resistance Transfer Factor" seen to have a decreased permeability for at least some of the antibiotics t which they are resistant (17) *Rosenkranz* (15) has found that this may indeed be true in the case of their resistance to streptomycin He found that ribosomes from "RTF-streptomycin resistant" cells are inhibited in their poly-U stimulated synthesis of polyphenylalanine by strepto mycin to the same extent as the ribosomes from streptomycin sensitive cells *Engelberg & Artman* (6) have shown that the uptake of strepto mycin was the same for streptomycin resistant as for sensitive cells, whereas the protein synthesis was inhibited in the sensitive but unaffected in the resistant cells by the presence of streptomycin

The resistance to streptomycin shown by cells carrying the Mu-factor may be due to a decreased permeability of the drug (12) Earlier reports from this laboratory have shown that cells carrying the Mu-factor in the extrachromosomal state are resistant to 200 µg of streptomycin per ml when growing in minimal medium, and less than 20 µg when in complete The "mutator-resistant" strain (11) resists more than 200 µg of streptomycin per ml also in complete medium (12) The present study is an attempt to see whether this difference is due to differences in the permeation of streptomycin under the various conditions The experiments have been based upon the technique by *Engelberg & Artman* (5, 6), by *Anand, Davis & Armitage* (1) and by *Plotz Dubin & Davis* (14)

MATERIALS AND METHODS

Bacterial strains K12 T71, T71Mu and T71Mu str r have previously been used in this laboratory (11, 12)

Media The minimal medium was prepared according to *Davis & Mingioli* (4) When complete medium was used in the present study the minimal medium was

be due to the microbe used, a streptomycin-dependent mutant of *E coli* Br Also, the results may have been influenced by the dense suspensions which have been employed when working with growing cells Preliminary experiments performed in our laboratory indicate a marked decrease in the uptake of ^{14}C-streptomycin with increasing density of the cell suspension

The strains T71Mu and T71Mu str r seem to have a smaller total uptake of ^{14}C streptomycin than the sensitive strain when growing in minimal medium, and also much less of the streptomycin is retained after washing with salt solution This is in agreement with our hypothesis of their resistance being due to a lack of penetration When growing in complete medium, T71Mu seems to take up the same proportion of the adsorbed streptomycin as when growing in minimal medium, but the total amount of the presumed internal streptomycin is greater In complete medium there is a much higher metabolic activity, and it seems that the streptomycin sensitivity of T71Mu in this medium is due to the fact that the amount of streptomycin which penetrates is sufficient to cause lethal interference on the faster working ribosomes The difference in the reaction to streptomycin of T71Mu in minimal medium versus a complete one may then be a question of quantity

The percentage of streptomycin retained by T71Mu str-r seems to be greater than that retained by T71Mu This was not expected since we know that streptomycin under these conditions causes no damage in the "mutator resistant" cells (12) One explanation may be that the high-level streptomycin resistance caused by the action of the mutator factor, probably by the attachment of this factor to the bacterial chromosome (11), is due to a change in the sensitivity of the ribosomes to streptomycin

SUMMARY

Evidence has been presented indicating that streptomycin is taken up by growing, sensitive populations of *E coli* K12 T71, and is bound in such a way as to resist exchange with inorganic ions

When the Mu-factor is present in the extrachromosomal state, the uptake of streptomycin is markedly decreased regardless of whether the cells are growing in minimal or in complete medium The particular low streptomycin resistance shown by these cells thus seems to be due to a lack of penetration

The streptomycin resistance shown by the "mutator resistant" cells may partly be due to a lack of penetration, and partly to another mechanism, possibly a change in the sensitivity of the ribosomes to streptomycin

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TABLE 2
Uptake of ^{14}C Streptomycin by Cells Growing in Complete Medium

Strain	Uptake of ^{14}C-strept µg/mg dry weight bact	Strept left after 4 wash. with salt sol
T71	4 40	2 07
T71 Mu	4 19	0 44
T71 Mu str r	4 29	0 93

Each result is the mean of 4 separate experiments

Under the conditions in which the experiments of Table 2 are performed, it has been found that T71 and T71Mu are rapidly killed, whereas T71Mu str-r will grow unaffectedly (12) The radioactivity measurements indicate no significant difference in the absorbtion of streptomycin to the three strains The proportion of radioactivity retained after washings with salt solution, however, seems to be very much like the one in the former set of experiments Around 50 per cent of the radioactive streptomycin is retained by the ordinary sensitive strain, around 10 per cent by the mutator strain, and approximately 20 per cent by the mutator streptomycin resistant strain

According to the experiments performed in other laboratories on the uptake of ^{14}C-streptomycin by *E coli* (6, 1) none of the radioactivity taken up by the cells is retained after washings with salt solution On the other hand, *Hancock* (13) found that growing cultures of *Bacillus megaterium* retained at least 50 per cent of the radioactive streptomycin after washings with salt solution Several experiments were performed in order to elucidate this problem with regard to the present system In our technique it proved impossible to completely remove the radioactive streptomycin from the strain T71 K12 by means of washings with salt solution Even 8 washings did not remove the radioactivity When the number of washings were increased, however, the deviation between the parallel experiments was great The amount of ^{14}C-streptomycin available limited further experimentation

DISCUSSION

Streptomycin exerts its killing effect by an interference with the functions of the ribosomes Working with growing, sensitive cells one would accordingly expect to find streptomycin inside the cell It might be suggested that intracellular streptomycin would resist washing with salt solution Thus we may distinguish between a total absorbtion of streptomycin to the cell and a presumed internal pool of streptomycin which can not be removed by washing with salt solution Such a hypothesis is in accordance with our results on growing, sensitive cells of *E coli* K12 T71, and also with the findings of *Hancock* (13) on *Bacillus megaterium* The different results of *Engelberg & Artman* (5, 6) may

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STUDIES ON TRANSFORMATION IN *MORAXELLA* AND ORGANISMS ASSUMED TO BE RELATED TO *MORAXELLA*

6 *A Distinct Group of* Moraxella nonliquefaciens-*Like Organisms (the "19116 51" Group)*

By

KJELL BOVRE

Received 31 vii 65

A homogeneous group of *Moraxella nonliquefaciens* strains has previously been examined by means of conventional tests (*Bovre* 1964 b, 1965 c) and in quantitative streptomycin resistance transformation (*Bovre* 1964 b, 1965 a, c) In parallel two deviating strains have been studied, one of which is the strain 19116 51 Growth in Koser s citrate medium, growth on Hugh & Leifson s medium and absence of nitrite production from nitrate distinguish the latter strain from the relatively fastidious and regularly nitrate reducing organisms considered to be representative *Moraxella nonliquefaciens* strains In repeated tests, strain 19116.51 has revealed a distinct deviation in terms of transformation from *Moraxella nonliquefaciens*, as here defined, and other moraxellae

It has been suggested (*Bovre* 1964 b) that strain 19116.51 might represent a distinct group of taxonomic interest Observations published a little later and independently by *Catlin & Cunningham* (1964), are very interesting from this point of view The latter authors found a first degree relationship in terms of dihydrostreptomycin resistance transformation between strain 19116.51 (which they had received from our institute before it had been examined genetically) and strains with the designation *Mima polymorpha* var *oxidans* They based their studies on a rather small number of assumed *Moraxella nonliquefaciens* strains (4 strains, of which 2 were primarily dihydrostreptomycin resistant and 1 was incompatible with the others) Assuming that strain 19116.51 is a representative *Moraxella nonliquefaciens* strain, they concluded that *Moraxella nonliquefaciens* and *Mima polymorpha* var *oxidans* (*De Bord* 1942) are identical and consequently discarded the latter term *Catlin* (1964) now names the strains in question *Moraxella nonliquefaciens*

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A homogeneous group of *Moraxella nonliquefaciens* strains has previously been examined by means of conventional tests (*Bøvre* 1964 b, 1965 c), and in quantitative streptomycin resistance transformation (*Bøvre* 1964 b, 1965 a, c) In parallel two deviating strains have been studied, one of which is the strain 19116 51 Growth in Koser's citrate medium growth on Hugh & Leifson's medium and absence of nitrite production from nitrate distinguish the latter strain from the relatively fastidious and regularly nitrate reducing organisms considered to be representative *Moraxella nonliquefaciens* strains In repeated tests, strain 19116.51 has revealed a distinct deviation in terms of transformation from *Moraxella nonliquefaciens*, as here defined, and other moraxellae

It has been suggested (*Bøvre* 1964 b) that strain 19116.51 might represent a distinct group of taxonomic interest Observations published a little later and independently by *Catlin & Cunningham* (1964), are very interesting from this point of view The latter authors found a first degree relationship in terms of dihydrostreptomycin resistance transformation between strain 19116.51 (which they had received from our institute before it had been examined genetically) and strains with the designation *Mima polymorpha* var *oxidans* They based their studies on a rather small number of assumed *Moraxella nonliquefaciens* strains (4 strains, of which 2 were primarily dihydrostreptomycin resistant and 1 was incompatible with the others) Assuming that strain 19116.51 is a representative *Moraxella nonliquefaciens* strain, they concluded that *Moraxella nonliquefaciens* and *Mima polymorpha* var *oxidans* (*De Bord* 1942) are identical and consequently discarded the latter term *Catlin* (1964) now names the strains in question *Moraxella nonliquefaciens*

The opinion of *Catlin & Cunningham* that strain 19116/51 is a representative *Moraxella nonliquefaciens* strain, is in conflict with the present author's own results The intention is now to establish that strain 19116/51 represents a distinct group of organisms The relationship of this group to *Moraxella nonliquefaciens* will be investigated in particular, and attempts will be made to evaluate differentiation by means of conventional criteria

MATERIAL AND METHODS

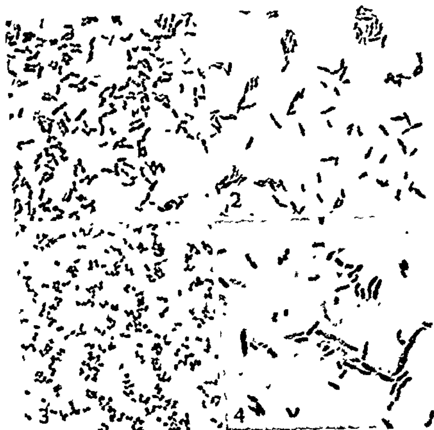
From Dr L O King, Communicable Disease Center, Georgia, U S A, were received a great number of unclassified oxidase ...
vn relations to
growth charac
In this way 8
4, *8232*, *9833* A608 and A1920) In
n strain 10973 was received from Dr
This is one of the *Mima polymorpha*
The 10th strain of the unclassified
material is the previously examined strain 19116/51
Strain A608 had
fl
th
to strain *152/52*
from strain 19116/
strains which grew luxuriant
ly on blood agar,
so excluded Most of the latter
strains had been designated ' typical *Mima polymorpha* var *oxidans* (*King* 1965) Three strains designated typical *Moraxella nonliquefaciens* (*King* 1965), were all completely examined by conventional means. They *were similar in* all respects to the group taken to represent *Moraxella nonliquefaciens* (*Bovre* 1964 b) and also revealed a first degree *affinity to the latter in* terms of streptomycin resistance transformation These three strains will not be further considered *in this paper*

The 10 above mentioned strains were examined in conventional tests as *described* in detail for *Moraxella nonliquefaciens* (*Bovre* 1964 b, 1965 c) According to the method used by *Kaffka* (1964) culture in the presence of low *concentrations of* penicillin was used to clarify the rod nature of some very coccoid strains

The degrees of genetic homogeneity of the 10 strains were expressed in terms of quantitative streptomycin resistance transformation The relations *in the latter* terms between the 10 strains and the group considered to represent *Moraxella nonliquefaciens* were subsequently examined Although the strain 19116/51 has been used previously in transformation experiments with *Moraxella bovis* (*Bøvre* 1965 a) reexamination was considered necessary to make certain that 500 µg of streptomycin per ml was not critically high for the selection of these eventual transformants The relations to the serum liquefying nonhaemolytic moraxellae were considered adequately determined with strain 19116/51 (*Bovre* 1965 c)

Transformation was performed according to the procedure in general use (*Bovre 1964 a* 1965 c), with controls as described Transforming DNA quantities were of the order 40 µg per ml With a view to experience obtained in transformations with the strains 19116/51 and 10973 (*Catlin & Cunningham* 1964) and with *Moraxella liquefaciens* (*Bovre* 1965 c) it was not relied upon transformant selection exclusively at 500 *µg of streptomycin* per ml although all DNAs of the present study were *prepared* from spontaneous mutants selected at 500 µg of streptomycin per ml Transformants were generally incubated for 6 7 days before assay

Observations on the strains 19116/51 and 10973 (*Catlin & Cunningham* 1964) led to the incubation of transformant assay plates at 29 30° C in some experiments in case even the usual temperature
Particular care was taken to elimi
cells in the least diluted parallels
phenotypic expression



Figs 1–4

… illin × 960

RESULTS

Description of the 10 Unclassified Strains

The microscopical picture of the strains was not uniform Some strains (5873, 8134, 19116 51 and A608) appeared as shown in Fig 1 *Their morphology* is in accordance with that of most *Moraxella nonliquefaciens* strains (*Bøvre* 1964 b) Other strains (5893, 8292 and *9833) had* a peculiar fusiform or lanceolate shape in the Gram stained smears (Fig 2) The remaining strains (5718, 10973 and A1920) *consisted of rather* small diplococci (Fig 3) Fig 4 shows the coccoid strain of Fig 3 after *culture in* the presence of penicillin in a concentration just below the minimum inhibitory concentration

Giant *forms* not induced by penicillin were often observed The strains were Gram-*negative, but* resistance to decolorization during

TABLE 1

Some Characteristics of the 10 Strains

Strain	Consistency of colonies	Agglutin ability in physiological saline	Growth on the surface of Hugh & Leifson's medium	Growth on citrate media	Nitrite production	Urease activity	Haemolysis	Serum lique faction
5718	Soft	—	+†	+§	+	—	—	—
5873	Soft	—	+	+	+	—	—	—
5893	Soft	—	+	+	+	—	—	—
8134	Soft	—	+	+	+	—	—	—
8292	Soft	—	+	+	+	—	—	—
9893	Almost coherent*	+	+	+	+	—	—	—
10973	Soft	—	+	+	—″	—	—	—
19116/51	Soft	—	+	+	—″	—	—	—
A608	Almost coherent*	+	+	(+)°	+	—	—	—
A1920	Soft	—	+	+	—″	—	—	—

* Consistency almost like that of *micrococci or gonococci* † + = *Fairly good growth* § + = *Weak growth in Koser's fluid* medium and on the same medium with agar added ° Questionable growth ability in Koser's fluid medium, but growth on the medium with agar added ″ Nitrate apparently not attacked, as shown by means of Zn powder reduction

Gram staining was often pronounced, as was irregular staining The microscopical picture seemed identical at 32 33° C and 37° C

The colonies on blood agar after incubation for 20 h were generally slightly smaller than those of *Moraxella nonliquefaciens* (0 8–1 2 mm) They were of the low conical type, with circular periphery and an even, glistening surface There was no pigmentation Their opacity was not *significantly more pronounced* than that of *Moraxella nonliquefaciens* A clear periphery of colonies could be observed in some strains The consistency of colonies was soft, with two exceptions (Table 1) All strains were nonhaemolytic Greenish discoloration of the blood agar was not observed

The two temperatures 32 33° C and 37° C seemed to stimulate growth equally well Growth was slightly retarded in a dry atmosphere at 37° C, as compared with growth in a humid atmosphere at the same temperature All strains failed to grow under strictly anaerobic conditions (hydrogen atmosphere) In 0 4 per cent Brain Heart Infusion agar stab culture all strains grew fairly well down to 5 mm below the surface

TABLE 2
Sensitivity to Antibiotics of the 10 Strains

Antibiotic	Range of inhibition zones in mm	Approximate range of minimum inhibitory concentrations (m i c.)*
Penicillin	33 41	0 07 – 0 007
Streptomycin	26 – 33	0 3 – 0 01
Chloramphenicol	33 – 40	0 3 – 0 05
Oxytetracycline	*28 34*	*0 2 – 0 02*
Erythromycin	29 – 40	0 5 – 0 02

Method of *Ericsson Hogman & Wickman* (1954) M i c values calculated from zone diameters by means of regression equations for each antibiotic (*Ericsson* 1960) M i c given as I U/ml for penicillin as µg ml for the other antibiotics

On solid 1 per cent peptone media growth was slightly better than that of *Moraxella nonliquefaciens* (*Bovre* 1965 c) Although the medium with Proteose Peptone no 3 (Difco) was inferior to other peptones also with these organisms, it generally supported growth Feeble growth was observed also in ordinary 1 per cent peptone water Fairly good growth was seen with all 10 strains on Hugh & Leifson's medium Growth on this medium was even better than that of *Moraxella bovis* and *Moraxella liquefaciens* (*Bovre* 1965 a, c) In Koser s citrate medium weak growth occurred with 9 strains On the same medium with agar added, all 10 strains grew feebly, but distinctly in daily subcultures for 7 days (Table 1)

The strains were all immotile, oxidase positive, and did not produce acid from glucose (tested on glucose ascites agar and on Hugh & Leifson s medium) There was no production of indol or hydrogen sulphide

TABLE 1

Some Characteristics of the 10 Strains

Strain	Consistency of colonies	Agglutin ability in physiological saline	Growth on the surface of Hugh & Leifson's medium	Growth on citrate media	Nitrite production	Urease activity	Haemolysis	Serum liquefaction
5718	Soft	–	+ †	+ §	+	–	–	–
5873	Soft	–	+	+	+	–	–	–
5893	Soft	–	+	+	+	–	–	–
8134	Soft	–	+	+	+	–	–	–
8292	Soft	–	+	+	+	–	–	–
9893	Almost coherent*	+	+	+	+	–	–	–
10973	Soft	–	+	+	–″	–	–	–
19116/51	Soft	–	+	+	–″	–	–	–
A608	Almost coherent*	+	+	(+)°	+	–	–	–
A1920	Soft	–	+	+	–″	–	–	–

* Consistency almost like that of micrococci or gonococci † + = Fairly good growth § + = Weak growth in Koser's fluid medium and on the same medium with agar added ° Questionable growth ability in Koser's fluid medium, but growth on the medium with agar added ″ Nitrate apparently not attacked, as shown by means of Zn powder reduction

TABLE 3

Quantitative Streptomycin Resistance Transformation between Members of the Presumptive "19116/51" Group

Recipient strain	Donor strain	Recipient count/ml	Interstrain trans formants/ml	Intrastrain trans formants/ml*	Ratio of inter to intrastrain transfor-mation
5873	5718	2 1 10^6	7 0 10^3(70)§	6 9 10^3(69)	1 0 10^0
	5893	1 2 10^7	4 6 10^3(46)	1 1 10^4(109)	4 2 10^{-1}
	8134	2 1 10^6	5 3 10^3(53)	6 9 10^3(69)	7 7 10^{-1}
	8292	1 2 10^7	5 3 10^3(53)	1 1 10^4(109)	4 9 10^{1}
	9893	2 1 10^6	5 0 10^3(50)	6 9 10^3(69)	7 2 10^{1}
	10973	2 1 10^6	5 4 10^3(54)	6 9 10^3(69)	7 8 10^{1}
	19116/51	2 1 10^6	5 2 10^3(52)	6 9 10^3(69)	7 7 10^{1}
	A608	2 1 10^6	6 1 10^3(61)	6 9 10^3(69)	8 8 10^{-1}
	A1920	2 1 10^6	5 0 10^3(50)	6 9 10^3(69)	7 2 10^{1}
8134	5718	1 8 10^7	1 3 10^4(130)	3 0 10^4(300)	4 3 10^{-1}
	5873	1 8 10^7	1 4 10^4(136)	3 0 10^4(300)	4 5 10^{-1}
	5893	1 2 10^6	1 5 $10^{[illegible]}$(15)	3 5 10^2(35)	4 3 10^{1}
	8292	1 2 10^6	2 3 $10^{[illegible]}$(23)	3 5 10^2(35)	6 6 10^{1}
	9893	1 8 10^7	1 0 10^4(103)	3 0 10^4(300)	3 4 10^{1}
	10973	1 8 10^7	1 3 10^4(130)	3 0 10^4(300)	4 3 10^{-1}
	19116/51	1 8 10^7	1 0 10^4(104)	3 0 10^4(300)	3 5 10^{1}
	A608	1 8 10^7	1 3 10^4(128)	3 0 10^4(300)	4 3 10^{-1}
	A1920	1 8 10^7	1 3 10^4(133)	3 0 10^4(300)	4 4 10^{1}
19119/51	5718	4 7 10^7	1 6 10^4(162)	3 0 10^4(302)	5 4 10^{-1}
	5873	4 7 10^7	2 2 10^4(222)	3 0 10^4(302)	7 4 10^{-1}
	5893	1 2 10^7	2 0 10^2(20)	5 5 10^2(55)	3 6 10^{1}
	8134	4 7 10^7	2 8 10^4(284)	3 0 10^4(302)	9 4 10^{-1}
	8292	1 2 10^7	4 7 10^2(47)	5 5 10^2(55)	8 5 10^{1}
	9893	Not counted	2 6 10^4(263)	3 0 10^4(296)	8 9 10^{-1}
	10973	9 0 10^7	4 2 10^3(42)	4 7 10^3(47)	8 9 10^{1}
	A608	Not counted	1 8 10^4(184)	3 0 10^4(296)	6 2 10^{1}
	A1920	Not counted	1 3 10^4(125)	3 0 10^4(296)	4 2 10^{-1}
A1920	5718	9 1 10^7	1 9 10^4(186)	2 6 10^4(259)	7 2 10^{1}
	5873	9 1 10^7	1 5 10^4(145)	2 6 10^4(259)	5 6 10^{-1}
	5893	7 3 10^6	2 5 10^2(25)	6 8 10^2(68)	3 7 10^{1}
	8134	9 1 10^7	1 7 10^4(172)	2 6 10^4(259)	6 6 10^{1}
	8292	7 3 10^6	3 7 $10^{[illegible]}$(37)	6 8 10^2(68)	5 4 10^{-1}
	9893	9 1 10^7	8 2 10^3(82)	2 6 10^4(259)	3 2 10^{1}
	10973	9 1 10^7	2 4 10^4(241)	2 6 10^4(259)	9 3 10^{-1}
	19116/51	9 1 10^7	2 4 10^4(242)	2 6 10^4(259)	9.3 10^{1}
	A608	9 1 10^7	9 6 10^3(96)	2 6 10^4(259)	3 7 10^{1}

Duration of DNA exposure 15 min Transformants selected at 50 μg of streptomycin per ml Incubation of transformants at 32–33° C

counted in simultaneous transformation Identical intrastrain transformant counts

of two plate counts Inoculum per plate

in simple or serum-enriched media Nitrate reduction tests gave identical results in simple and serum-enriched medium The latter results and some other characteristics are presented in Table 1. The results of tests for fastidiousness and of biochemical tests were identical at 32–33° C and 37° C

The wild type strains were all strongly sensitive to common antibiotics (Table 2)

Transformation Reactions

The ratios of inter- to intrastrain transformation frequencies were determined between the 10 unclassified strains, using 4 recipients and the whole material as donors against each recipient The results are collected in Table 3 There is a uniform distribution of interstrain transformation frequencies close to simultaneously assayed frequencies of intrastrain transformation The resulting ratios of inter- to intrastrain transformation are ranging from 3.2×10^{-1} to 1.0×10^{0} The transformants obtained in the experiments of Table 3 were all resistant to 1000 μg of streptomycin per ml, as shown by velvet replica plating of transformant assay plates

From now on the 10 unclassified strains will be designated the "19116/51" group

In Table 4 are presented results of quantitative streptomycin resistance transformation with 3 recipients from the "19116/51" group and 5 donors of *Moraxella nonliquefaciens* The ratios of inter- to intrastrain transformation in the most sensitive experiment are below 2.4×10^{-5} The somewhat higher limits obtained in other experiments are considered due to the lower efficiency of simultaneously performed intrastrain transformation In an additional quantitative experiment (not tabulated) with the recipient strain 19116/51 and selection of transformants at 10 μg of streptomycin per ml, the *Moraxella nonliquefaciens* donors 178/62, 270/60, 3828/60 and 4235/62 were also ineffective (ratios of inter- to intrastrain streptomycin resistance transformation below 1.7×10^{-4})

In the quantitative experiments presented in Table 5, all strains of the "19116/51" group were acting as donors on 2 *Moraxella nonliquefaciens* recipients The ratios of inter- to intrastrain transformation in the most sensitive experiment are shown to be below 2.5×10^{-5} Other sensitive experiments reveal upper limits of similar order for the relation between *Moraxella nonliquefaciens* and the "19116/51" group in terms of streptomycin resistance transformation

Parallel to most reactions in Tables 4 and 5 simultaneous exposure to the respective heterologous DNAs without termination of transformation with DNase (continuous DNA exposure), was applied In these parallels very low-frequent transformation reactions were observed between the "19116/51" group and *Moraxella nonliquefaciens*, regardless of the group serving as source of the recipient These reactions will be de-

TABLE 3

Quantitative Streptomycin Resistance Transformation between Members of the Presumptive 19116/51" Group

Recipient strain	Donor strain	Recipient count/ml	Interstrain trans formants/ml	Intrastrain trans formants/ml*	Ratio of inter to intrastrain transfor mation
5873	5718	2 1 10^{6}	7 0 10^{3}(70)§	6 9 10^{3}(69)	1 0 10^{0}
	5893	1 2 10^{7}	4 6 10^{3}(46)	1 1 10^{4}(109)	4 2 10^{-1}
	8134	2 1 10^{6}	5 3 10^{3}(53)	6 9 10^{3}(69)	7 7 10^{-1}
	8292	1 2 10^{7}	5 3 10^{3}(53)	1 1 10^{4}(109)	4 9 10^{-1}
	9893	2 1 10^{6}	5 0 10^{3}(50)	6 9 10^{3}(69)	7 2 10^{-1}
	10973	2 1 10^{6}	5 4 10^{3}(54)	6 9 10^{3}(69)	7 8 10^{-1}
	19116/51	2 1 10^{6}	5 2 10^{3}(52)	6 9 10^{3}(69)	7 7 10^{-1}
	A608	2 1 10^{6}	6 1 10^{3}(61)	6 9 10^{3}(69)	8 8 10^{-1}
	A1920	2 1 10^{6}	5 0 10^{3}(50)	6 9 10^{3}(69)	7 2 10^{-1}
8134	5718	1 8 10^{7}	1 3 10^{4}(130)	3 0 10^{4}(300)	4.3 10^{-1}
	5873	1 8 10^{7}	1 4 10^{4}(136)	3 0 10^{4}(300)	4 5 10^{-1}
	5893	1 2 10^{6}	1 5 10^{2}(15)	3 5 10^{2}(35)	4 3 10^{-1}
	8292	1 2 10^{6}	2 3 10^{7}(23)	3 5 10^{2}(35)	6 6 10^{-1}
	9893	1 8 10^{7}	1 0 10^{4}(103)	3 0 10^{4}(300)	3 4 10^{-1}
	10973	1 8 10^{7}	1 3 10^{4}(130)	3 0 10^{4}(300)	4 3 10^{-1}
	19116/51	1.8 10^{7}	1 0 10^{4}(104)	3 0 10^{4}(300)	3 5 10^{-1}
	A608	1 8 10^{7}	1 3 10^{4}(128)	3 0 10^{4}(300)	4 3 10^{-1}
	A1920	1 8 10^{7}	1 3 10^{4}(133)	3 0 10^{4}(300)	4 4 10^{-1}
19119/51	5718	4 7 10^{7}	1 6 10^{4}(162)	3 0 10^{4}(302)	5 4 10^{-1}
	5873	4 7 10^{7}	2 2 10^{4}(222)	3 0 10^{4}(302)	7 4 10^{-1}
	5893	1 2 10^{7}	2 0 10^{2}(20)	5 5 10^{2}(55)	3 6 10^{-1}
	8134	4 7 10^{7}	2 8 10^{4}(284)	3 0 10^{4}(302)	9 4 10^{-1}
	8292	1 2 10^{7}	4 7 10^{2}(47)	5 5 10^{2}(55)	8 5 10^{-1}
	9893	Not counted	2 6 10^{4}(263)	3 0 10^{4}(296)	8 9 10^{-1}
	10973	9 0 10^{7}	4 2 10^{3}(42)	4 7 10^{3}(47)	8 9 10^{-1}
	A608	Not counted	1 8 10^{4}(184)	3 0 10^{4}(296)	6 2 10^{-1}
	A1920	Not counted	1 3 10^{4}(125)	3 0 10^{4}(296)	4 2 10^{-1}
A1920	5718	9 1 10^{7}	1 9 10^{4}(186)	2 6 10^{4}(259)	7 2 10^{-1}
	5873	9 1 10^{7}	1 5 10^{4}(145)	2 6 10^{4}(259)	5 6 10^{-1}
	5893	7 3 10^{6}	2 5 10^{2}(25)	6 8 10^{2}(68)	3 7 10^{-1}
	8134	9 1 10^{7}	1 7 10^{4}(172)	2 6 10^{4}(259)	6 6 10^{-1}
	8292	7 3 10^{6}	3 7 10^{2}(37)	6 8 10^{2}(68)	5 4 10^{-1}
	9893	9 1 10^{7}	8 2 10^{3}(82)	2 6 10^{4}(259)	3 2 10^{-1}
	10973	9 1 10^{7}	2 4 10^{4}(241)	2 6 10^{4}(259)	9.3 10^{-1}
	19116/51	9 1 10^{7}	2 4 10^{4}(242)	2 6 10^{4}(259)	9 3 10^{-1}
	A608	9 1 10^{7}	9 6 10^{3}(96)	2 6 10^{4}(259)	3 7 10^{-1}

* [illegible] indicate parallel experiments

§ Figures in brackets indicate means of two plate counts Inoculum per plate 0 1 ml of the appropriate dilution

scribed and compared with other reactions of similar order in a separate report

The results of two-way transformation experiments with the "19116/51" group and *Moraxella bovis*, are presented in Table 6 The ratio of inter- to intrastrain transformation between the two is below 2 0 10^{-4} in the most sensitive experiment In parallel continuous exposure to heterologous DNA only a few resistant colonies arose on each plate, indicating that the ratio most probably is considerably lower than the limit obtained quantitatively (see *Bovre* 1964 b, 1965 b)

In the intrastrain reactions of Tables 4, 5 and 6 the counts of transformants did not differ significantly when selected at 10, 50 or 500 µg of streptomycin per ml, and means of plate counts were calculated collectively for the different selection concentrations

TABLE 4

Quantitative Streptomycin Resistance Transformation with Recipients of the '19116/51 Group and Moraxella nonliquefaciens *Donors*

Recipient strain	Donor strain of *M nonliq*	Recipient count/ml	Interstrain trans formants/ml	Intrastrain trans formants/ml*	Ratio of inter to intrastrain transfor mation
5873	7784	1 8 10^{8}	<10^{1}(0)§	4 1 10^{5}(205)	<2 4 10^{-5}
	4663/62	1 8 10^{8}	<10^{1}(0)	4 1 10^{5}(205)	<2 4 10^{-5}
	4626/62	4 5 10^{8}	<10^{1}(0)	2 0 10^{5}(204)	<4 9 10^{-5}
	5050/62	4 5 10^{8}	<10^{1}(0)	2 0 10^{5}(204)	<4 9 10^{-5}
	13536/62	4 5 10^{8}	<10^{1}(0)	2 0 10^{5}(204)	<4 9 10^{-5}
19116/51	7784	9 0 10^{7}	<10^{1}(0)	5 1 10^{4}(51)	<2 0 10^{-4}
	4663/62	9 0 10^{7}	<10^{1}(0)	5 1 10^{4}(51)	<2 0 10^{-4}
A1920	7784	4 5 10^{7}	<10^{1}(0)	6 5 10^{4}(65)	<1 5 10^{-4}
	4663/62	4 5 10^{7}	<10^{1}(0)	6 5 10^{4}(65)	<1 5 10^{-4}
	5050/62	4 5 10^{7}	<10^{1}(0)	6 5 10^{4}(65)	<1 5 10^{-4}
	13536/62	4 5 10^{7}	<10^{1}(0)	6 5 10^{4}(65)	<1 5 10^{-4}

Duration of DNA exposure 15 min Transformants selected at 10 50 and 500 µg of streptomycin per ml

* Intrastrain transformants have been counted in simultaneous transformation of the recipient with its own mutant DNA

§ Figures in brackets indicate means of 3–5 plate counts (collectively for different transformant selection concentrations) Inoculum per plate 0 1 ml of the appropriate dilution

All donor mutants of the "19116/51" group were uniformly resistant to 1000 µg of streptomycin per ml, as shown by means of velvet replica plating of large numbers of colonies The donors of *Moraxella nonliquefaciens* and *Moraxella bovis* also had this high degree of streptomycin resistance (*Bovre* 1964 b, 1965 a)

The lack of measurable compatibility of the "19116/51" group and the group considered to represent *Moraxella nonliquefaciens*, was con-

TABLE 5

Quantitative Streptomycin Resistance Transformation with Moraxella nonliquefaciens Recipients and Donors of the 19116/51 Group

Recipient strain of *M nonliq*	Donor strain	Recipient count/ml	Interstrain transformants/ml	Intrastrain transformants/ml*	Ratio of inter to intrastrain transformation
4 84	5873	2.2 × 10^{8}	$<10^{1}$(0)§	4.0 × 10^{5}(40)	$<2.5 \times 10^{-5}$
	8134	2.2 × 10^{8}	$<10^{1}$(0)	4.0 × 10^{5}(40)	$<2.5 \times 10^{-5}$
	8292	2.2 × 10^{8}	$<10^{1}$(0)	4.0 × 10^{5}(40)	$<2.5 \times 10^{-5}$
	19116/51	2.2 × 10^{8}	$<10^{1}$(0)	4.0 × 10^{5}(40)	$<2.5 \times 10^{-5}$
	10943	7.5 × 10^{7}	$<10^{1}$(0)	2.4 × 10^{5}(237)	$<4.2 \times 10^{-5}$
4663/62	5718	8.0 × 10^{7}	$<10^{1}$(0)	3.5 × 10^{5}(35)	$<2.9 \times 10^{-5}$
	5873	1.5 × 10^{7}	$<10^{1}$(0)	6.5 × 10^{4}(65)	$<1.5 \times 10^{-4}$
	5893	8.0 × 10^{7}	$<10^{1}$(0)	3.5 × 10^{5}(35)	$<2.9 \times 10^{-5}$
	9893	8.0 × 10^{7}	$<10^{1}$(0)	3.5 × 10^{5}(35)	$<2.9 \times 10^{-5}$
	10973	8.0 × 10^{7}	$<10^{1}$(0)	3.5 × 10^{5}(35)	$<2.9 \times 10^{-6}$
	19116/51	9.8 × 10^{7}	$<10^{1}$(0)	2.3 × 10^{5}(226)	$<4.4 \times 10^{-5}$
	A608	8.0 × 10^{7}	$<10^{1}$(0)	3.5 × 10^{5}(35)	$<2.9 \times 10^{-5}$
	A1920	8.0 × 10^{7}	$<10^{1}$(0)	3.5 × 10^{5}(35)	$<2.9 \times 10^{-5}$

Duration of DNA exposure 15 min Transformants selected at 10 50 and 500 µg of streptomycin per ml

* Intrastrain transformants have been counted in simultaneous transformation of the recipient with its own mutant DNA

§ Figures in brackets indicate means of 3 6 plate counts (collectively for different transformant selection concentrations) Inoculum per plate 0.1 ml of the appropriate dilution

TABLE 6

Quantitative Streptomycin Resistance Transformation between Moraxella bovis and Members of the 19116/51 Group

Recipient strain	Donor strain	Recipient count/ml	Interstrain transformants/ml	Intrastrain transformants/ml*	Ratio of inter to intrastrain transformation
M bovis 10900	10973	1.2 × 10^{8}	$<10^{1}$(0)§	2.3 × 10^{4}(23)	$<4.3 \times 10^{-4}$
	19116/51	1.2 × 10^{8}	$<10^{4}$(0)	2.3 × 10^{4}(23)	$<4.3 \times 10^{-4}$
19116/51	*M bovis* 10900	9.0 × 10^{7}	$<10^{1}$(0)	5.1 × 10^{4}(51)	$<2.0 \times 10^{-4}$

Duration of DNA exposure 15 min Transformants selected at 10 50 and 500 µg f streptomycin per ml

Intrastrain transformants have been counted in simultaneous transformation of the recipient with its own mutant DNA

§ Figures in brackets indicate means of 5–8 plate counts (collectively for different transformant selection concentrations) Inoculum per plate 0.1 ml of the appropriate dilution.

scribed and compared with other reactions of similar order in a separate report

The results of two-way transformation experiments with the "*19116/51*" group and *Moraxella bovis*, are presented in Table 6. The ratio of inter- to intrastrain transformation between the two is below $2.0 \cdot 10^{-4}$ in the most sensitive experiment. In parallel continuous exposure [illegible] on each plate, [illegible] er than the limit obtained quantitatively (see *Bovre* 1964 b, 1965 b)

In the intrastrain reactions of Tables 4, 5 and 6 the counts of transformants did not differ significantly when selected at 10, 50 or 500 μg of streptomycin per ml, and means of plate counts were calculated collectively for the different selection concentrations

TABLE 4

Quantitative Streptomycin Resistance Transformation with Recipients of the '19116/51' Group and Moraxella nonliquefaciens *Donors*

Recipient strain	Donor strain of *M nonliq*	Recipient count/ml	Interstrain trans-formants/ml	Intrastrain trans-formants/ml*	Ratio of inter to intrastrain transformation
5873	7784	$1.8 \cdot 10^{8}$	$<10^{1}(0)$§	$4.1 \cdot 10^{5}(205)$	$<2.4 \cdot 10^{-5}$
	4663/62	$1.8 \cdot 10^{8}$	$<10^{1}(0)$	$4.1 \cdot 10^{5}(205)$	$<2.4 \cdot 10^{-5}$
	4626/62	$4.5 \cdot 10^{8}$	$<10^{1}(0)$	$2.0 \cdot 10^{5}(204)$	$<4.9 \cdot 10^{-5}$
	5050/62	$4.5 \cdot 10^{8}$	$<10^{1}(0)$	$2.0 \cdot 10^{5}(204)$	$<4.9 \cdot 10^{-5}$
	13536/62	$4.5 \cdot 10^{8}$	$<10^{1}(0)$	$2.0 \cdot 10^{5}(204)$	$<4.9 \cdot 10^{-5}$
19116/51	7784	$9.0 \cdot 10^{7}$	$<10^{1}(0)$	$5.1 \cdot 10^{4}(51)$	$<2.0 \cdot 10^{-4}$
	4663/62	$9.0 \cdot 10^{7}$	$<10^{1}(0)$	$5.1 \cdot 10^{4}(51)$	$<2.0 \cdot 10^{-4}$
A1920	7784	$4.5 \cdot 10^{7}$	$<10^{1}(0)$	$6.5 \cdot 10^{4}(65)$	$<1.5 \cdot 10^{-4}$
	4663/62	$4.5 \cdot 10^{7}$	$<10^{1}(0)$	$6.5 \cdot 10^{4}(65)$	$<1.5 \cdot 10^{-4}$
	5050/62	$4.5 \cdot 10^{7}$	$<10^{1}(0)$	$6.5 \cdot 10^{4}(65)$	$<1.5 \cdot 10^{-4}$
	13536/62	$4.5 \cdot 10^{7}$	$<10^{1}(0)$	$6.5 \cdot 10^{4}(65)$	$<1.5 \cdot 10^{-4}$

Duration of DNA exposure 15 min. Transformants selected at 10, 50 and 500 μg of streptomycin per ml

* Intrastrain transformants have been counted in simultaneous transformation of the recipient with its own mutant DNA

§ Figures in brackets indicate means of 3-5 plate counts (collectively for different transformant selection concentrations). Inoculum per plate 0.1 ml of the appropriate dilution

All donor mutants of the "19116/51" group were uniformly resistant to 1000 μg of streptomycin per ml, as shown by means of velvet replica plating of large numbers of colonies. The donors of *Moraxella nonliquefaciens* and *Moraxella bovis* also had this high degree of streptomycin resistance (*Bovre* 1964 b, 1965 a)

The lack of measurable compatibility of the "*19116/51*" group and the group considered to represent *Moraxella nonliquefaciens*, was con-

TABLE 5

Quantitative Streptomycin Resistance Transformation with Moraxella nonliquefaciens *Recipients and Donors of the 19116/51 Group*

Recipient strain of *M nonliq*	Donor strain	Recipient count/ml	Interstrain transformants/ml	Intrastrain transformants/ml*	Ratio of inter to intrastrain transformation
7784	5873	2.2 × 10^{8}	< 10^{1}(0)§	4.0 × 10^{5}(40)	< 2.5 × 10^{-5}
	8134	2.2 × 10^{8}	< 10^{1}(0)	4.0 × 10^{5}(40)	< 2.5 × 10^{-5}
	8292	2.2 × 10^{8}	< 10^{1}(0)	4.0 × 10^{5}(40)	< 2.5 × 10^{-5}
	19116/51	2.2 × 10^{8}	< 10^{1}(0)	4.0 × 10 (40)	< 2.5 × 10^{-5}
	10973	7.5 × 10^{7}	< 10^{1}(0)	2.4 × 10^{5}(237)	< 4.2 × 10^{-5}
4663 62	5718	8.0 × 10^{7}	< 10^{1}(0)	3.5 × 10^{5}(35)	< 2.9 × 10^{-5}
	5873	1.5 × 10^{7}	< 10^{1}(0)	6.5 × 10^{4}(65)	< 1.5 × 10^{-4}
	5893	8.0 × 10^{7}	< 10^{1}(0)	3.5 × 10^{5}(35)	< 2.9 × 10^{-5}
	9893	8.0 × 10^{7}	< 10^{1}(0)	3.5 × 10^{5}(35)	< 2.9 × 10^{-5}
	10973	8.0 × 10^{7}	< 10^{1}(0)	3.5 × 10^{5}(35)	< 2.9 × 10^{-5}
	19116/51	9.8 × 10^{7}	< 10^{1}(0)	2.3 × 10^{5}(226)	< 4.4 × 10^{-5}
	A608	8.0 × 10^{7}	< 10^{1}(0)	3.5 × 10^{5}(35)	< 2.9 × 10^{-5}
	A1920	8.0 × 10^{7}	< 10^{1}(0)	3.5 × 10^{5}(35)	< 2.9 × 10^{-5}

Duration of DNA exposure 15 min Transformants selected at 10, 50 and 500 µg of streptomycin per ml

* Intrastrain transformants have been counted in simultaneous transformation of the recipient with its own mutant DNA

§ Figures in brackets indicate means of 3–6 plate counts (collectively for different transformant selection concentrations) Inoculum per plate 0 1 ml of the appropriate dilution

TABLE 6

Quantitative Streptomycin Resistance Transformation between Moraxella bovis *and Members of the 19116/51 Group*

Recipient strain	Donor strain	Recipient count/ml	Interstrain trans formants/ml	Intrastrain trans-formants/ml*	Ratio of inter to intrastrain transfor mation
M bovis 10900	10973	1.2 × 10^{8}	< 10^{1}(0)§	2.3 × 10^{4}(23)	< 4.3 × 10^{-4}
	19116/51	1.2 × 10^{8}	< 10^{1}(0)	2.3 × 10^{4}(23)	< 4.3 × 10^{-4}
19116/51	*M bovis* 10900	9.0 × 10^{7}	< 10^{1}(0)	5.1 × 10^{4}(51)	< 2.0 × 10^{-4}

Duration of DNA exposure 15 min Transformants selected at 10, 50 and 500 µg of streptomycin per ml

* Intrastrain transformants have been counted in simultaneous transformation of the recipient with its own mutant DNA

§ Figures in brackets indicate means of 5–8 plate counts (collectively for different transformant selection concentrations) Inoculum per plate 0 1 ml of the appropriate dilution

trolled by various means. The selection of eventual intergroup transformants at 29–30° C was tried in quantitative experiments with the strains 19116/51 and A1920 as recipients, and in experiments with all strains of the "19116/51" group acting as donors. These attempts all failed as regards detection of transformants. In some instances the intrastrain and interstrain (intergroup) transformation mixtures both were assayed for transformants undiluted and diluted to the same extent. In no case it was possible to observe "drowning" of intrastrain transformants due to a heavy inoculum. Neither could such effect be obtained with prolongation of the phenotypic expression period, within reasonable limits. Interstrain (intergroup) transformants did not occur in the diluted parallels or on plates with shorter expression period. Thus, no indication was observed that the absence of intergroup transformants in quantitative experiments was due to an artifact.

DISCUSSION AND CONCLUSION

The essential outcome of this part of the studies is the existence of a "19116/51" group of organisms very similar to *Moraxella nonliquefaciens* (as here defined), but deviating from the latter when the test system is somewhat extended. The test for nitrite production from nitrate is not generally distinctive between the two groups. On the other hand, the less pronounced fastidiousness of the "19116/51" group is considered clearly substantiated. Fairly good growth on Hugh & Leifson's medium and growth on the citrate media employed distinguish the group from *Moraxella nonliquefaciens*. It might be suggested that the strains of the "19116/51" group are more adapted to laboratory conditions than the recently isolated *Moraxella nonliquefaciens* strains with which they have been compared. However, one of the strains which *Catlin & Cunningham* (1964) found genetically compatible with strain 19116/51 (strain 7146/51) had been examined by *Henriksen* (1952) shortly after isolation. He observed moderate growth of this strain in the defined medium of *Audureau* (1940). This medium consists of ethyl alcohol, citrate, ammonia and inorganic salts.

Further comparative biochemical, serological and perhaps also morphological studies of the two groups would be of interest. Ecological studies might be of particular value. Unfortunately, the source of only two strains of the present "19116/51" group is known. They had been isolated from blood and cerebrospinal fluid, respectively. Of the five strains found compatible with strain 19116/51 by *Catlin & Cunningham* (1964), the source of three is known. Two were of genitourinary origin and one had been isolated from a leg ulcer. For comparison, no strain recently isolated from the nose cavity belonged to the "19116/51" group (*Bovre* 1964 b).

The primary indication that the "19116/51" group is a distinct entity, is its homogeneity in terms of streptomycin resistance transformation

and its uniform deviation in these terms from *Moraxella nonliquefaciens* and other moraxellae The homogeneity of the "19116.51" group is expressed by ratios of inter- to intrastrain transformation which are consistent with a first degree relationship (*Bøvre* 1964 b) Its relations to *Moraxella bovis* and the serum-liquefying, nonhaemolytic moraxellae, as well as its relation to *Moraxella nonliquefaciens*, seem to be more distant than the interrelations between entities recognized as *Moraxella* species (*Bøvre* 1965 a, c)

With great probability, the conclusion of *Catlin & Cunningham* (1964) that *Mima polymorpha* var *oxidans* (*De Bord* 1942) and *Moraxella nonliquefaciens* are identical must have been based on reactions *within* the "19116.51" group Consistent with this is also that they practically failed in attempts at transformation of their strains with *Moraxella bovis* and *Moraxella liquefaciens* DNAs There is still no indication that a strain named *Mima polymorpha* var *oxidans* has close relation in terms of streptomycin resistance transformation to *Moraxella nonliquefaciens* strains considered representative (*Bøvre* 1964 b)

The discovery of the "19116.51" group of strains may be an important step towards clarification of affinities in the vicinity of *Moraxella* and *Mima polymorpha* var *oxidans*

ADDENDUM

The source of 5 more strains of the "19116.51" group has been made available by Dr E O King The strain 5718 had been isolated from a scalp lesion, strain 5873 from cerebrospinal fluid, strain 5893 from chest fluid, strain 8292 from blood and strain 9893 from nose

SUMMARY

Previous studies have shown the deviating behaviour of strain 19116.51, as compared with a large group of strains considered to be typical *Moraxella nonliquefaciens* and with other recognized moraxellae

The present study, involving the strain 19116.51 and 9 other strains, revealed the existence of a distinct "19116.51" group of *Moraxella nonliquefaciens* like organisms This group was homogeneous in terms of quantitative streptomycin resistance transformation (ratios of inter- to intrastrain transformation ranging from 1/3 to 1, approximately) In sensitive quantitative tests the "19116.51" group had no compatibility in terms of streptomycin resistance transformation with presumptively representative *Moraxella nonliquefaciens* strains (ratios of inter- to intrastrain transformation being less than $2.4 \cdot 10^{-3}$)

The main distinctive trait found in conventional tests was the clearly less pronounced fastidiousness of the "19116.51" group than of *Moraxella nonliquefaciens*

The discovery of the "19116.51" group is considered of potential

trolled by various means. The selection of eventual intergroup transformants at 29–30° C was tried in quantitative experiments with the strains 19116/51 and A1920 as recipients, and in experiments with all strains of the "19116/51" group acting as donors. These attempts all failed as regards detection of transformants. In some instances the intrastrain and interstrain (intergroup) transformation mixtures both were assayed for transformants undiluted and diluted to the same extent. In no case it was possible to observe "drowning" of intrastrain transformants due to a heavy inoculum. Neither could such effect be obtained with prolongation of the phenotypic expression period, within reasonable limits. Interstrain (intergroup) transformants did not occur in the diluted parallels or on plates with shorter expression period. Thus, no indication was observed that the absence of intergroup transformants in quantitative experiments was due to an artifact.

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The primary indication that the "19116/51" group is a distinct *entity*, is its homogeneity in terms of streptomycin resistance *transformation*

Acta path et microbiol scandinav 65, 653, 1965

BRIEF REPORT

ABO BLOOD-GROUPS IN PARKINSON'S DISEASE

By *R R Strang*

The following study was carried out to analyse the ABO blood group distribution of patients with Parkinsonism An investigation of this type has not been previously reported for this disease

The 245, statistically evaluated patients [illegible] and in many cases they had been referr[illegible] to the geographical distribution, and th[illegible] post encephalitic or Idiopathic paralysis [illegible] selected, and representative one No case [illegible] the study

There were 130 males and 115 females in the Swedish material, ranging in age from 37 to 72 years The control figures were obtained from the paternity cases of *Beckman*'s survey (1), which he concluded to be the most reliable for statistical purposes

Two small groups of patients from Norway and Finland are also included in the table Percentage figures only, are shown for these patients, and the control distributions are obtained from the studies of *Hartmann & Lundewall* (2) and *Eriksson et al* (3)

TABLE 1

ABO Blood-Group Distribution of Swedish, Finnish and Norwegian Patients with Parkinson's Disease

Patient Material	A	B	AB	O	Total
Observed Swedish patients	100	22	10	113	245
Beckman's Survey patients	4939	1010	504	3974	10457
Expected Parkinson distribution from above	115	25	12	93	245
Observed Finnish patients	7	3	1	6	17
Percentage Finnish patients	41	18	6	35	100
Control percentage (*Eriksson et al*)	43	16	8	33	100
Observed Norwegian patients	9	2	1	9	21
Percentage Norwegian patients	43	9	5	43	100
Control percentage (*Hartmann & Lundewall*)	49	8	4	39	100

[illegible] of the control

References 1 *Beckman, L* Thesis Uppsala, Sweden 1959 — 2 *Hartmann, O & Lundewall J* Thesis Oslo 1944 — 3 *Eriksson, A W, Frisk, M D & Nevanlinna, H R* Acta genet 12 312-321, 1962

Received 13 x 65 from *Parkinson Research Unit Karolinska Sjukhuset, Stockholm, Sweden*

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TABLE 1

Antigenic Composition of his *Recombinants from Crosses between a* his *T1 Recipient (S paratyphi B SH 1120) and* his^+ *Smooth Donors SW 1444 (S abony 4 12*) and SH 713 (S montevideo 6 7)*

	strain	antigens	no	genetic markers§ leu	his	aro	str	met	4% NaCl	agglutination in anti T1	anti 4	anti 9, 12	anti 6 7
recipient	SH 1120	T1		+	—	+	s	+	—	+	—	—	—
cross 1													
donor	SW 1444	4 12*			+	—	r	—	—	—	+	+	
his^+ recombinants		T1 4 12*	39		+	+	s	+	—	+	+	+	
(total 42)		T1	3		+	+	s	+	—	+	—	—	
cross 2													
donor	SH 713	6 7		—	+				—	—			+
his recombinants		T1 6 7	13	+	+				—	+			+
(total 18)		T1	5	+	+				—	+			—

* The presence of antigen 5 was not tested in all cases and is disregarded

§ Loci for *leu* — leucine *his* — histidine *aro* — phenylalanine and tyrosine, *met* — methionine biosynthesis + — synthetic ability, — — inability *str* — locus for streptomycin resistance s — sensitivity r — resistance

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BRIEF REPORT

THE PRODUCTION, BY RECOMBINATION, OF *SALMONELLA* FORMS WITH BOTH T1 AND O SPECIFITIES

By *Matti Sarvas & P. Helena Mäkelä*[1]

The somatic O antigen of *Salmonella* is affected by the so-called S-T variation the T form has a new antigen T in place of the specific O antigen of the S (smooth) form The T form is in many respects intermediate between S and R (rough) forms
, and smooth in culture
O antigen of the strain
specificity T1 has been encountered as a natural
variant of
variant of groups B, E, G, R, S, and T of
Salmonella
1960) A comparative chemical analysis
of the som
own that the T as well as R forms are
totally lacking in the O specific monosaccharides of the S forms (*Lüderitz et al* 1960) T1 lipopolysaccharide differs from that of R forms in having an appreciably higher content of ribose and galactose, these two sugars probably play a role in T1 specificity as they are able to inhibit T1 hemagglutination (*Lüderitz et al* 1966)

Most of the genes participating in the determination of *Salmonella* O-specificity reside in a region of the chromosome known as the *O*-locus closely linked to *his* the locus for histidine biosynthesis (*Johnson et al* 1965, *Mäkelä* 1965, 1966) In order to find out whether this locus is also concerned with the determination of T specificity we made genetic crosses between S and T forms by means of sexual recombination (*Mäkelä* 1963, 1965)

The recipient bacteria in the crosses were a T1 form of *Salmonella paratyphi B* originally obtained from Prof Kauffmann, State Serum Institute, Copenhagen, its *his*⁺ mutant SH 1120 was used *Hfr* mutants of *S abony* (O antigens 4, 5, 12 as in *S paratyphi*) and of *S montevideo* (O-antigens 6, 7) were used as donors Because of the known close linkage between the *O* locus and *his*, the recombinants which had inherited the *his* allele of the donor were selected Most of them were expected to have inherited the donor *O*-locus as well All recombinants were tested by slide agglutination, and the results are recorded in Table 1

Ninety to 70 per cent of the *his*⁺ recombinants proved to have the donor O-specificities 4 12 (cross 1, *S abony* donor) or 6 7 (cross 2 *S montevideo* donor) However, all 60 recombinants also agglutinated in anti-T1 unlike either donor Thus the majority of *his*⁺ recombinants appeared to be antigenically T1, 4 12 (cross 1) or T1, 6, 7 (cross 2) This antigenic constitution was stable during several subcultures and single colony isolations precluding a transient diploid state The above formula was confirmed by absorption of known anti T1, anti-4, 12 and anti 6 7 sera (Table 2) It thus seems that the gene(s) determining antigen T1 cannot be allele(s) of the *O* locus

The results seem to indicate that the lack of O specificity in bacteria known as T forms is unrelated to the presence of T specificity In other words, the T and the S forms differ in two respects firstly, the T form has active gene(s) for the production of T1 antigen, which are lacking or inactive in the S form secondly the T form lacks O-specificity through a *rou* mutation in a locus controlling the production of the antigenic lipopolysaccharide In the T1 S recombinants the mutant *rou*-gene has been replaced by an intact *O* locus without affecting the status of the *T1 gene(s)*, their genotype would thus be T1 0 4 12 or T1, 0–6 7, while the original T form is *T1, rou*

"Mixed" T-S *Salmonella* forms have not been found in nature although a similar form was selected from *S weslaco* (O antigen 42) by *Schlosshardt* (1960) It seems

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INDEX

VOL. 65 FASC. 1-4 1965

possible that they would have been overlooked in diagnostic bacteriology since they agglutinate like normal S forms in specific anti O sera It is also known that the T form is unstable (*Kauffmann* 1956)

TABLE 2

Absorption of anti T1 anti 4 12 and anti 6 7 Sera by Parent Strains and Representative Recombinants of Crosses 1 and 2 (Table 1)

The strain used for absorption	Reciprocals of serum titers		
	T1 serum tested with T1 bacteria	anti 4 12 serum tested with 4 12 bacteria	anti 6 7 serum tested with 6 7 bacteria
—	128	160	16
SH 1120 (T1 recipient)	4	80	16
SW 1444 (4 5 12 donor)	64	0	
SH 713 (6 7 donor)	64		0
Recombinants†			
cross 1 (T1 4 12)	8	0	
cross 1 (T1 6 7)	8		0

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INDEX

VOL 65 FASC 1–4 1965

SUPPLEMENTA

Supplementum 176 *Nystrom, Stig H. M.* A study on supratentorial meningiomas. With special reference to gross and fine structure. Pp. 90, 1965

Supplementum 177 *Madsen, Arve* Studies on basal-cell epithelioma of the skin. Pp. 63, 1965

Supplementum 178 *Ernström, Ulf* Studies on growth and cytomorphosis in the thymo-lymphatic system. With special reference to the influence of the thymus and the thyroid in guinea pigs. Pp. 38, 1965

Supplementum 179 *Brun, Arne* The subpial granular layer of the foetal cerebral cortex in man. Its ontogeny and significance in congenital cortical malformations. Pp. 98, 1965

SUPPLEMENTA

Supplementum 176 *Nyström Stig H M* A study on supratentorial meningiomas With special reference to cross and fine structure Pp 90 1965

Supplementum 177 *Madsen Arne* Studies on basal-cell epithelioma of the skin Pp 63 1965

Supplementum 178 *Ernström Ulf* Studies on growth and cytomorphosis in the thymo lymphatic system With special reference to the influence of the thymus and the thyroid in guinea pigs Pp 38 1965

Supplementum 179 *Brun Arne* The subpial granular layer of the foetal cerebral cortex in man Its ontogeny and significance in congenital cortical malformations Pp 98 1965